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VOLATILE ACID PRODUCTION OF "S. LACTICUS" AND THE ORGANISMS ASSOCIATED WITH IT IN STARTERS

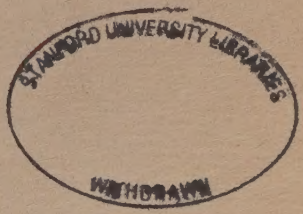
A DISSERTATION

SUBMITTED TO THE FACULTY
OF THE OGDEN GRADUATE SCHOOL OF SCIENCE
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

DEPARTMENT OF HYGIENE AND BACTERIOLOGY

BY

BERNARD WERNICK HAMMER



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VOLATILE ACID PRODUCTION OF *S. LACTICUS* AND THE ORGANISMS ASSOCIATED WITH IT IN STARTERS

By B. W. Hammer

The organisms ordinarily classed as *S. lacticus* (or *Bact. lactis acidi*) represent a group that is extremely important in the field of dairying. Altho they are responsible for a great deal of loss, since they are the principal type that sours milk and cream and thus spoils for certain purposes large quantities of these materials, they are ordinarily classed as the desirable lactic acid bacteria because of the role they play in the making of certain dairy products.

In the manufacture of butter, cheese and fermented milks, acid-producing bacteria are ordinarily added to the cream or milk in the form of starter. The statement has frequently been made that starters are pure cultures of the lactic acid bacteria, but data have recently been published by different workers showing that there are organisms other than *S. lacticus* present in starters and that these exert a very important influence on the development of aroma and flavor. It accordingly seems well established that, to be satisfactory, a starter should contain more than one type of organism.

The aroma of a desirable starter is definitely acid and, since lactic acid is odorless, this must be due to acids other than lactic. *S. lacticus* produces only a comparatively small amount of volatile acid, but when other organisms present in starters are grown in combination with it, a volatile acidity is developed that is essentially the same as that produced by good starters. Accordingly, it seems evident that the organisms other than *S. lacticus* present in starters are important from the standpoint of the volatile acidity and thus also of the aroma and probably the flavor that are secured.

The present paper deals with the volatile acidity produced, both alone and in combination, by *S. lacticus* and the organisms associated with it in starters.

HISTORICAL

The addition of a natural starter to boiled milk intended for churning was described as early as 1776 [Heinemann, (20)]. In 1887 a description of the method of using "lange wei" as a starter for Edam cheese was published in a pamphlet by Boekel [Henzold (21)]; the starter was supposed to exert a favorable effect on cheese defects and especially to prevent swelling. Buttermilk from a churning of good butter was apparently extensively used for starting the acid fermentation in cream in-

tended for buttermaking before the development of artificial cultures.

Conn, working at the Storrs Agricultural Experiment Station, began in 1889 the publication of a series of reports on cream ripening. He early concluded (4) that the ripening of cream is a complex matter and that, "while the ripening of cream is undoubtedly dependent upon the presence of bacteria, it is doubtful whether one species can produce what is known as ripened cream." This investigator (5) specifically pointed out that in cream ripening something besides souring takes place and that, "while the addition of acid does certainly hasten the souring, it does not accomplish the other purposes of ripening, for the whole process is a complicated one connected with bacterial growth and the formation of acid is only a part of it." Conn studied the effect of various organisms on the flavor of butter. Of twenty species whose effects were reported in 1893 (7), only three produced strikingly bad results, while the rest gave good butter or had no marked influence; no single species produced a typical ripening of the cream or the usually expected flavor in the butter and the few acid-producing species did not have as good an influence on the butter as the alkali-producing species. Later Conn (8) described his culture Bacillus No. 41, which was isolated from milk from Uruguay, and stated that it improved the score of butter. Farrington and Russell (15 and 16), however, did not get an improvement in the quality of the butter when they used the organism describd by Conn.

Conn (10) pointed out that acid and flavor should be distinguished and that, while the acid is developed from the sugar, the flavor probably comes from some other source; he also considered that aroma should be separated from flavor and stated that it is more unusual for an organism to produce aroma than flavor.

The use of the so-called pure cultures for the ripening of cream was the result of the investigations of Storch (29), who studied organisms from sources such as buttermilk, butter and ripened cream and found that different acid producing forms induced very different flavors and aromas. From completely ripened cream secured at a plant known for the full aroma of its butter, he isolated seven acid producing bacteria, of which two produced in milk on souring a very aromatic odor, while one of these yielded as full an aroma (if not in as high a degree) as a full-soured cream in the dairy possessed. Butter made from pasteurized cream with pure cultures had a very normal, clean, mild, acid flavor, so there was no doubt but that these bacteria are one of the sources of butter aroma.

Weigmann (34), who studied the influence of the acid bacteria, also found a variation in the products formed; when he secured

a strong aroma he did not have a clean tasting butter of good keeping quality and when he had a clean tasting butter of good keeping quality he did not have a strong aroma, so he suggested the use of mixtures of the two kinds of organisms or an attempt to find cultures in which the qualities of the two were combined.

According to Orla-Jensen (25), the first commercial starter cultures were put out in 1890. Following their introduction there appeared a large number of reports dealing with their use under practical conditions, but containing little if any information on the development of flavor and aroma.

Weigmann (35), in answering an inquiry, stated that the question as to whether lactic acid bacteria themselves produce the aroma of butter or whether in addition other bacteria are important was not settled, altho the results and trials spoke for the first assumption.

In 1894 the European dairy papers (e. g. Milch-Ztg. 1894, 23 p. 687) reported the isolation by Biaille of the aroma-producing material of Isigny butter; this was sold under the name of Bouquet d' Isigny.

The culture of Lorentz [Vieth (33)] was unusual in that, according to the manufacturer, it contained, together with the lactic acid bacteria, *Oidium lactis*, which was designated as an aroma organism.

By 1897 [Hansen (18)], the use of bacterial cultures was very common in Denmark. Of 866 dairies in that country, 802 used the cultures.

Conn (11) following statements that had been made in Europe, pointed out that the so-called pure cultures are not all alike and that while most are pure cultures, some are mixtures of different organisms and that one culture contained no less than ten or twelve different species mixed together.

Altho at this time the superiority of Danish butter was commonly attributed to the use of pure cultures, the idea was also prevalent that the influence of pasteurization was much greater than that of the cultures.

McDonnell (23) classified the lactic acid bacteria and designated one type as *Bact. lactis acidi aromaticum*; the author stated that in milk this organism produced, when not over ripened by high temperatures, a mild, acid, weak, aromatic taste and that the dairies used cultures of it for cream ripening.

Evans, Hastings and Hart (14) found that "No cheddar flavor is obtained in pasteurized milk cheese when the organisms of the *Bacterium lactis acidi* group alone are used as starters." This indicates that *Bact. lactis acidi* is limited in its flavor-producing power.

Sammis and Bruhn (26) have, however, reported a method for the manufacture of cheddar cheese from pasteurized milk.

In New Zealand the use of pasteurized milk for cheese making is on a satisfactory basis [Stevenson and Grant (28)]; for the season ending March 31, 1919,* out of a total production of 57,654 tons, 29,036 tons were made from pasteurized milk.

Hart, Hastings, Flint, and Evans (19) found that representatives of the coccus group and organisms isolated from cheddar cheese produced large quantities of volatile acids, particularly acetic acid, when grown in milk, and suggested that since these organisms are present in large numbers in cheese, they produce much of the volatile fatty acids developing during ripening.

Evans (13) studied the streptococci concerned in the ripening of cheese and presented data showing that *S. lacticus* produces only a small quantity of acetic acid. This investigator called attention to the conclusion of Leichmann (22) that *S. lacticus* produced no volatile acid and to the findings of Orla-Jensen (24) and others that small quantities of volatile acid, chiefly acetic, are produced by this organism. Evans reported two organisms, *Strep. x* and *Strep. kefir*, as producing much larger amounts of volatile acid than *S. lacticus*.

Hammer and Bailey (17) in a study of starters found *S. lacticus* producing amounts of volatile acid which were in agreement with the amounts reported by Evans (13) for this organism, but which were much smaller than the amounts produced by good starters. They showed that in the starters studied there was an organism which produced little if any change in appearance in a tube of litmus milk but which, when grown with *S. lacticus*, produced a volatile acidity essentially the same as that produced by good starters; this organism in pure culture also produced considerable amounts of volatile acid.

Storch (30) reported the presence in starters of organisms (which he called *x* bacteria) that played an important part in the production of a good starter.

Boekhout and Ott De Vries (1) isolated organisms which gave in combination with lactic acid forms the characteristic aroma of starters.

METHODS USED

The methods used were essentially the same as those employed by Hammer and Bailey (17).

The volatile acidity was determined by distilling with steam a 250 gram portion of the milk after the addition of 15 cc. of approximately n/1 H_2SO_4 until 1000 cc. of distillate were secured, after which the distillate was titrated with n/10 NaOH, using phenolphthalein as an indicator. Usually five grams of

* Letter from Hon. D. Cuddie, Dir. of Dairy Div. Dept. Agr. Industries and Commerce, N. Z.

Na_2SO_4 were used in the milk to aid in keeping down foaming. The results obtained were expressed as the cc. of n/10 NaOH required; the value given accordingly means the number of cc. of n/10 NaOH required to neutralize the first liter of distillate obtained when a 250 gram portion of milk was distilled with steam after the addition of 15 cc. of approximately n/1 H_2SO_4. The results cannot be considered as representing all of the volatile acidity produced, since only 1000 cc. of distillate were secured, and it is probable that the percentage of the total volatile acid obtained by the method is only approximately constant, because of the variations in the acids present in the different samples of milk. However, the method was considered to yield comparative results and to be satisfactory for the work for which it was intended.

The milk used for the volatile acid determinations was skim milk sterilized in the autoclave in 325 cc. portions with an exposure of 10 to 15 pounds for from 20 to 30 minutes. The containers used were for the most part wide-mouthed bottles or flasks stoppered with cotton. When the milk was distilled before inoculation by the method outlined above, the first liter of distillate usually required from 1.7 to 2.8 cc. of n/10 NaOH for its neutralization. In a few instances it was necessary to discard a lot of milk because of an excessive value for the uninoculated material.

The total acidity of the milk was usually determined by titrating 20 grams with n/10 NaOH, using phenolphthalein and calculating as the percent lactic acid.

RESULTS OBTAINED

THE TYPES OF ORGANISMS ASSOCIATED WITH *S. LACTICUS* IN *STARTERS*

In the work reported by Hammer and Bailey (17), an organism was found associated with *S. lacticus* in starters which produced little if any change in the appearance of litmus milk, but which in combination with *S. lacticus* produced a volatile acidity comparable with the volatile acidity produced by a good starter. This organism will be referred to as associated organism Type A. In studying more in detail the volatile acid production of the starter organisms, it seemed advisable to determine whether or not organisms important from the standpoint of volatile acid production, other than the one reported by Hammer and Bailey, could be found often enough in the starters available to suggest that they were important.

Organisms were isolated from a considerable number of starters from a variety of sources, and studied. One of the commercial cultures that was frequently examined usually contained an organism, often in considerable numbers, which was

quite different from Type A but which also produced large amounts of volatile acid. In general the volatile acidity produced by this organism when grown alone was greater than the volatile acidity produced by Type A alone. The organism also produced a definite reddening in litmus milk, usually without coagulation, altho a small amount of curd in the bottom of the container, or even complete coagulation, was occasionly observed. It further differed from Type A in that it gave a definite growth at 37°C. while Type A did not. Accordingly this organism has been considered as representing a form distinct from Type A and will be referred to as Type B.

Type B was occasionally found in commercial cultures other than the one from which it was first secured and was also found in natural starters. It was, however, repeatedly met with in only the one commercial culture and this, which showed the Type B organism in nearly all of a considerable number of tests extending over a period of about two years, seemed to differ decidedly from all other cultures studied in the character of its associated organisms. Type B was sometimes found in the same culture with Type A, especially in natural starters.

Organisms other than the two mentioned, capable of producing a high volatile acidity in milk, have been found, but these have not been isolated often enough from the starters studied to indicate for them an important role in the ripening of starters. However, the finding of such organisms suggests that there may be starters in which organisms other than Types A and B are important. It seems, however, inadvisable to deal with these under the head of starter organisms until starters have been found in which they are of significance.

In the following discussion the two types of organisms associated with *S. lacticus* are considered separately.

INVESTIGATIONS WITH TYPE A

ISOLATION OF THE ASSOCIATED ORGANISM OF THE A TYPE

Two general methods have been rather extensively used for the isolation of Type A from starter: First, plating and picking off colonies, and second, the elimination of other organisms, especially *S. lacticus*, by repeated transfers on whey agar slopes. A third method, consisting of transferring at various times from a tube of starter to both milk and a whey agar slope until the milk tube showed no change while the slope showed growth, was also used, but only in a few cases, as it did not seem to offer any advantages. This method was later found to have been employed by Storch (30).

Plate Method: Whey agar, which is a very satisfactory medium for the growth of Type A, was the principal one used

in the isolations by the plate method, but beef infusion agar was found satisfactory and would undoubtedly have done equally as well; beef extract agar is not well adapted to the growth of starter organisms and probably should be avoided. Plates were poured from a starter after suitable dilution and incubated at a temperature of about 20° C. for from two to four days. A plate giving a good distribution of colonies was selected and a considerable number of colonies picked. The colonies were either put on whey agar slopes or into litmus milk. If they were put on agar slopes an inoculation was made from each into a tube of litmus milk as soon as a good growth was secured and the cultures which produced little or no change were reserved for study. When the colonies were picked into litmus milk the tubes showing little or no change were cultured on agar slopes to be sure that an organism had been recovered from the plates and to have agar cultures available for further study. Putting the colonies into litmus milk required less media than putting them on agar, since the litmus milk eliminated the *S. lacticus* colonies at once, while the agar slopes did not; but inoculating on agar first had the advantage of detecting at once the instances in which there was a failure to secure organisms from the original plate. Agar slopes inoculated from a colony on an agar plate were also somewhat more satisfactory for future study than agar slopes inoculated from milk cultures, since the latter sometimes yielded only a comparatively few colonies and in these cases it was necessary to smear these colonies out in order to get abundant inoculating material. When a growth on whey agar which produced little or no change in litmus milk was secured, it was tested out for volatile acid production in combination with *S. lacticus*. The cultures secured were sometimes purified in the usual way; but this does not seem necessary ordinarily since, the contaminating organism is likely to be *S. lacticus* which is detected by the preliminary tests.

Repeated Transfer: By smearing a small amount of starter on a whey agar slope and carrying the growth thus secured thru a number of transfers on the same medium at a temperature approximating 20° C., it was often possible to obtain a growth which, when inoculated into litmus milk, produced little or no change. This is the method used by Hammer and Bailey (17) in their early attempts to secure the associated organism. The growth obtained was then tried out for volatile acid production. Previous to this, it was sometimes purified by plating, but this was not usually necessary, since *S. lacticus* is the organism whose elimination is most difficult and the method insures the absence of this type. In a few instances the repeated transfer method failed to yield the associated organism in pure culture, even after as many as twenty transfers on whey agar slopes.

Often, however, Type A was obtained in six to ten transfers and sometimes in less. Table I presents the data obtained in connection with some of the isolations that were quickly successful; from these results it is evident that long intervals, such as 7 to 11 days between transfers, yield cultures of Type A with a smaller number of inoculations than do shorter intervals.

The repeated transfer method of isolation undoubtedly depends on the somewhat more rapid growth of Type A than of *S. lacticus,* which results in a gradual elimination of the latter organism. The difference in the rate of growth of the organisms is apparently not the same with all combinations of the two, since the associated type is more easily isolated from some starters than from others by the repeated transfer method.

Transferring at Various Times from a Starter: With this method a tube of starter was held and transfers made at various times to both litmus milk and a whey agar slope. Ordinarily, after several weeks a set of transfers was secured in which the litmus milk showed no change, while the whey agar slope contained colonies. Table II gives the data obtained in connection with the isolation by this method of two cultures that represent the extremes encountered from the standpoint of the time required for *S. lacticus* to die out.

The method depends on the more rapid death in old acid milk of *S. lacticus* than of Type A. From unreported results obtained it is evident that, unless the transfers are rather frequently made, the period when *S. lacticus* is dead and Type A alive may be missed. In some cases the number of colonies of Type A obtained on the whey agar slope at the time *S. lacticus* was dead was very small. These results suggest that the difference in the resistance of the two organisms to the conditions encountered in acid milk is not very great.

TABLE I. ISOLATION OF ASSOCIATED ORGANISM (TYPE A) BY THE REPEATED TRANSFER METHOD
ROOM TEMPERATURE INCUBATION

Trial No.	Date of Transfer from Starter to Whey Agar Slope	Transfer 2		Transfer 3		Transfer 4		Transfer 5		Transfer 6	
		Date	Change produced when growth was inoc. into litmus milk	Date	Change produced when growth was inoc. into litmus milk	Date	Change produced when growth was inoc. into litmus milk	Date	Change produced when growth was inoc. into litmus milk	Date	Change produced when growth was inoc. into litmus milk
1	10-1	10-4	coag.	10-7	coag.	10-10	coag.	10-14	coag.	10-17	none
2	11-4	11-6	coag.	11-10	coag.	11-13	coag.	11-19	none	11-27	none
3	1-6	1-17	coag.	1-24	none	1-31	none				
4	2-10	2-17	coag.	2-24	none	3-3	none				
5	2-21	2-28	coag.	3-7	coag.	3-15	none				

TABLE II. ISOLATION OF ASSOCIATED ORGANISM (TYPE A) BY METHOD OF TRANSFERRING FROM A STARTER AT VARIOUS TIMES
ROOM TEMPERATURE INCUBATION

Trial No.	Date starter was put away	Date of first successful transfers	Time in days required to isolate
1	3-3	3-25	22
2	12-13	2-10	59

Testing out the Organism Secured: After securing a good growth on whey agar which produced little or no change in litmus milk, it was tested out by growing in sterile milk (1) alone and (2) in combination with a culture of *S. lacticus* which previous trials had shown produced only a low volatile acidity, and then determining the volatile acid production. If the value for the organisms in combination was definitely higher than for the organism under test when grown alone and approximated the value obtained with a good starter, the culture was considered to be of the associated type.

PRESENCE OF TYPE A IN STARTERS BEING CARRIED IN CREAMERIES

The associated organism of the A type was found in many samples of starter that were being carried under practical conditions, the isolations being made by both the plate and the repeated transfer methods. The organism was found so regularly that it would seem, even without any of the other available proof, that it must be of importance. In a very few instances an attempt to isolate Type A by the plate method was unsuccessful, but this was in all probability due to picking too few colonies; in some of the isolation work only a small number were studied. It seems from the results obtained that in the creamery starters examined, Type A was almost always present. It was constantly found in an excellent starter which had been carried in a creamery for about sixteen years and with which many tubs of high grade butter had been made.

PRESENCE OF TYPE A IN COMMERCIAL STARTERS

A considerable number of commercial cultures were examined for Type A, since the presence of this organism in creamery starters is no assurance of its presence in the commercial cultures used, because of the possibility of infection in the creamery. The method of examination was to plate milk cultures direct and to plate after one or more transfers in sterile milk in the case of powdered cultures. A number of colonies were picked from each set of plates and some of the cultures showing no change in litmus milk were tried out for volatile acid production alone and in combination with *S. lacticus* cultures from the same plates; the volatile acid production of the *S. lacticus* cultures alone was also determined.

The general type of results obtained is shown in table III; these data show the usual low volatile acidity for *S. lacticus*, a higher volatile acidity for the associated organism (Type A) and the highest value for the two in combination.

In the examination of commercial cultures, organisms of the A type were found in the product put out by most manufacturers. The culture prepared by one concern gave Type A in only a portion of the tests made, but it almost always yielded Type B, while that of another manufacturer failed in certain instances at least to give either type. In one trial this latter product showed 72 *S. lacticus* cultures from 72 colonies picked from a whey agar plate poured from the fifth transfer in sterile

TABLE III. VOLATILE ACID PRODUCTION OF *S. LACTICUS* AND ASSOCIATED ORGANISM (TYPE A) ISOLATED FROM COMMERCIAL CULTURES
ROOM TEMPERATURE INCUBATION

Manufacturer	Type of culture	Inoculation	Total acidity in percent	Volatile acidity
A	Milk	*S. lacticus* 1	.76	5.6*
		Associated Organism 1	.24	13.5
		S. lacticus 1 plus Associated Organism 1	.86	35.5
		S. lacticus 2	.79	4.5
		Associated Organism 2	.24	21.3
		S. lacticus 2 plus Associated Organism 2	.83	35.8
		S. lacticus 3	.77	3.7
		Associated Organism 3	.25	15.7
		S. lacticus 3 plus Associated Organism 3	.76	34.7
A	Powder	*S. lacticus* 1	.77	4.4
		Associated Organism 1	.24	13.2
		S. lacticus 1 plus Associated Organism 1	.90	32.5
		S. lacticus 2	.76	3.5
		Associated Organism 2	.24	13.0
		S. lacticus 2 plus Associated Organism 2	.82	31.3
		S. lacticus 3	.75	5.0
		Associated Organism 3	.23	13.8
		S. lacticus 3 plus Associated Organism 3	.75	31.0
B	Powder	*S. lacticus* 1	.91	4.3
		Associated Organism 1	.25	14.3
		S. lacticus 1 plus Associated Organism 1	1.01	33.5
		S. lacticus 2	.87	4.6
		Associated Organism 2	.25	14.2
		S. lacticus 2 plus Associated Organism 2	1.10	36.4
C	Milk	*S. lacticus* 1	.92	6.6
		Associated Organism 1	.28	17.8
		S. lacticus 1 plus Associated Organism 1	1.12	30.5
		S. lacticus 2	.95	9.0
		Associated Organism 2	.34	19.8
		S. lacticus 2 plus Associated Organism 2	1.12	36.2
		S. lacticus 3	.95	6.2
		Associated Organism 3	.32	17.2
		S. lacticus 3 plus Associated Organism 3	1.08	41.3
D	Powder	*S. lacticus* 1	.96	2.2
		Associated Organism 1	.25	14.5
		S. lacticus 1 plus Associated Organism 1	1.14	31.2
		S. lacticus 2	.78	4.2
		Associated Organism 2	.27	18.6
		S. lacticus 2 plus Associated Organism 2	.74	31.3
		S. lacticus 3	.79	4.3
		Associated Organism 3	.25	19.0
		S. lacticus 3 plus Associated Organism 3	.85	31.8

* As explained under "Methods Used," these values represent the cc. of n/10 NaOH required for the neutralization of the first liter of distillate obtained when 250 grams of milk were distilled with steam after the addition of 15 cc. of approximately n/1 H_2SO_4.

TABLE IV. VOLATILE ACID PRODUCTION OF COMMERCIAL CULTURES, FREE FROM TYPE B, AFTER VARIOUS TRANSFERS IN STERILE MILK

ROOM TEMPERATURE INCUBATION

Mfgr. A powdered culture		Mfgr. D powdered culture		Mfgr. E powdered culture		Mfgr. F culture powdered		Mfgr. F powdered culture		Mfgr. G powdered culture		Mfgr. G powdered culture	
Transfer No.	Volatile acidity	Transfer No.	Volatile acidity	Transfer No.	Volatile acidity	Transfer No.	Volatile acidity	Transfer No.	Volatile acidity	Transfer No.	Volatile acidity	Transfer No.	Volatile acidity
3	39.1	3	42.2	3	11.4	3	40.0	2	37.5	3	34.0	1	20.7
6	40.5	6	40.4	6	9.8	6	37.4	4	42.0	6	35.0	2	31.7
								5	39.1			3	35.3
												5	34.6

milk. More conclusive proof that this culture contained no associated organisms was secured by running volatile acid determinations on the first and fourth transfers in sterile milk; the values obtained were 10.3 and 7.7. Additional data on a culture from the same source (manufacturer E) are given in table IV, which shows the volatile acidity produced by commercial cultures, known to be free from Type B, after various numbers of transfers in sterile milk. These results, which were all obtained on powdered cultures, are further evidence that commercial cultures contain Type A since in general the volatile acidities are entirely too high to have been produced by *S. lacticus* alone.

It is probable that the cultures containing neither of the associated organisms are put out that way intentionally, because of the frequent statements that starters are pure cultures of lactic acid organisms; in the absence of tests as to the quality of starters made from such cultures the idea might be held that they are actually superior to others. In dairy establishments these cultures do not necessarily give unsatisfactory results, since they are in all probability quickly contaminated with the associated organisms. In a number of trials pure cultures of *S. lacticus* were soon contaminated when carried under practical conditions by an experienced buttermaker, so that the starter yielded a high volatile acidity. Storch (30) found the same contamination to occur in Denmark.

INOCULATION OF *S. LACTICUS* FROM ONE SOURCE AND TYPE A FROM ANOTHER

It seemed desirable to determine whether or not a certain *S. lacticus* and Type A combination producing a high volatile acidity was a specific one, or if any organism belonging to the A type would produce a high volatile acidity in combination with any *S. lacticus* culture. Accordingly cultures of Type A were inoculated with *S. lacticus* cultures from starters other than those from which the Type A cultures were secured and

the volatile acidity determined after a satisfactory incubation period. The results obtained are presented in table V; the *S. lacticus* cultures used had all been found (many of them repeatedly) to produce only the usual low volatile acidity.

From table V it is evident that cultures of Type A (of which there were 15, representing 7 different starters from four manufacturers) produced high volatile acidities in 42 trials when grown in combination with *S. lacticus* cultures from starters other than those from which the associated organisms came. The volatile acidities of the combinations varied from 28.7 to 45.1, with the great majority running between 30.0 and 40.0, and agreed with those secured when the associated organisms were grown with *S. lacticus* cultures from the same starter. The results indicate that Type A from one starter gives the same general results with *S. lacticus* cultures from other starters as it does with *S. lacticus* cultures from the starter from which it came.

The volatile acidity produced by Type A isolated from varous starters, in combination with cultures of *S. lacticus* secured from sources other than starters, was determined with 14 cultures of *S. lacticus* (13 were isolated from sour cream and one was purchased from a concern selling cultures). The results secured, which include the volatile acidities of the *S. lacticus* cultures alone, are presented in table VI. The data show that a high volatile acidity (from 30.0 to 42.4), which is essentially the same as that obtained when the associated organism is grown in combination with cultures of *S. lacticus* isolated from starters, was secured in all cases; the *S. lacticus* cultures alone gave the usual low volatile acidity. These results indicate that the combined

TABLE V. VOLATILE ACID PRODUCTION SECURED WHEN THE ASSOCIATED ORGANISM (TYPE A) FROM ONE STARTER WAS GROWN WITH *S. LACTICUS* FROM OTHERS ROOM TEMPERATURE INCUBATION

Trial No.	Volatile acidity	Trial No.	Volatile acidity
22	38.5	1	28.7
23	39.9	2	35.0
24	45.1	3	38.8
25	31.0	4	38.1
26	39.6	5	34.3
27	36.5	6	37.0
28	35.1	7	44.7
29	34.6	8	40.7
30	34.5	9	34.3
31	43.0	10	30.4
32	30.6	11	28.8
33	35.9	12	35.7
34	35.8	13	41.0
35	44.3	14	35.3
36	37.2	15	35.1
37	35.8	16	33.7
38	39.5	17	33.3
39	39.6	18	35.6
40	34.0	19	35.9
41	35.5	20	35.7
42	37.9	21	34.7

TABLE VI. VOLATILE ACID PRODUCTION SECURED WHEN ASSOCIATED ORGANISMS (TYPE A) WERE GROWN WITH *S. LACTICUS* CULTURES FROM SOURCES OTHER THAN STARTER

ROOM TEMPERATURE INCUBATION

S. lacticus culture No.	Source of *S. lacticus*	Assoc. Org. No.	Total acidity in percent		Volatile acidity	
			S. lacticus alone	The two combined	*S. lacticus* alone	The two combined
1	Sour cream	1	1.01	1.12	8.1	36.9
1		2		1.18		41.9
1		3		1.19		38.3
2	Sour cream	1	1.05	1.13	9.5	36.7
2		2		1.17		35.2
3	Sour cream	1	1.02	1.17	12.5	33.8
3		3		1.09		39.3
4*	Sour cream	1	.61	.77	7.3	42.4
5*	Sour cream	1	.64	.81	5.0	34.9
6*	Sour cream	3	.68	.82	5.8	35.5
7*	Sour cream	3	.64	1.03	5.6	36.1
8*	Sour cream	2	.59	.63	5.3	32.1
9	Sour cream	3	.87	.98	7.6	32.7
10	Sour cream	2	.88	1.05	6.1	31.7
11	Sour cream	1	.90	.96	8.8	32.4
12	Sour cream	1	1.06	1.19	7.0	35.8
13	Sour cream	2	1.04	1.17	7.0	31.2
14	Purchased	1	.87	.92	9.1	37.8
14		2		1.00		30.0
14		3		1.04		32.5

* Slow curdler.

action of *S. lacticus* and the associated organism is not characteristic of the *S. lacticus* cultures from starters, but can also be obtained with cultures of this organism from sources such as sour cream. The data cannot be considered to show that all cultures of *S. lacticus* act in the same way when grown with the associated organism, since the number of cultures was small, but the indications are that many of them do.

From the data presented in tables V and VI it is evident that there is nothing specific about the Type A and *S. lacticus* combination, but that a Type A culture yields a high volatile acidity in combination with *S. lacticus* cultures from a variety of sources. The situation here is quite different than in the case of the ropy condition in milk due to the combined action of *S. lacticus* and another organism; in studying such a case, Buchanan and Hammer (3) found that only certain cultures of *S. lacticus* yielded the ropy condition when grown in combination with the other organism.

MECHANISM OF THE HIGH VOLATILE ACID PRODUCTION WITH TYPE A

The data which have been presented in tables III to VI, as well as those previously reported by Hammer and Bailey (17), show that the associated organism (Type A) and *S. lacticus* in combination produce a higher volatile acidity than does either

TABLE VII. VOLATILE ACID PRODUCTION SECURED WHEN THE ASSOCIATED ORGANISM (TYPE A) WAS INOCULATED FIRST AND *S. LACTICUS* LATER
ROOM TEMPERATURE INCUBATION

Inoculation	Date of Inoc. Assoc. Org.	Date of Inoc. *S. lacticus*	Date Examined	Total acidity in percent	Volatile acidity
Assoc. Org. 1 alone	5/2		5/7	.27	18.3
Assoc. Org. 1 plus *S. lacticus* A	5/2	5/3	5/8	1.08	39.9
Assoc. Org. 1 plus *S. lacticus* A	5/2	5/5	5/12	.95	41.3
Assoc. Org. 3 alone	5/2		5/7	.27	23.9
Assoc. Org. 3 plus *S. lacticus* C	5/2	5/3	5/8	.95	42.8
Assoc. Org. 3 plus *S. lacticus* C	5/2	5/5	5/12	1.00	39.4
Assoc. Org. 4 alone	5/2			.28	23.8
Assoc. Org. 4 plus *S. lacticus* D	5/2	5/3	5/8	.96	43.1
Assoc. Org. 4 plus *S. lacticus* D	5/2	5/5	5/12	.92	40.2
Assoc. Org. 1 alone	5/13		5/21	.24	21.6
Assoc. Org. 1 plus *S. lacticus* A	5/13	5/15	5/19	1.10	40.6
Assoc. Org. 1 plus *S. lacticus* A	5/13	5/17	5/21	1.08	37.2
Assoc. Org. 4 alone	5/13		5/21	.26	20.7
Assoc. Org. 4 plus *S. lacticus* D	5/13	5/15	5/20	1.00	46.2
Assoc. Org. 4 plus *S. lacticus* D	5/13	5/17	5/21	.96	32.6

organism alone. In order to secure information on the mechanism of the high volatile acid production when the organisms are grown together, experiments were carried out to determine more definitely the influence of each on this volatile acid production. The cultures of *S. lacticus* used were all known to produce the usual low volatile acidity.

Table VII presents the results obtained when the associated organism (Type A) was inoculated first and *S. lacticus* later, and, for comparison, the results obtained with the associated organism alone. The data show that when the associated organism was inoculated first and *S. lacticus* at a later date, up to four days at least, a high volatile acidity (from 32.6 to 46.2) was secured. This volatile acidity was much greater than the volatile acidity obtained with the associated organism alone (from 18.3 to 23.9) and was essentially the same as that secured with a good starter.

In order to determine whether or not thermostable products of the associated organism would stimulate the production of a high volatile acidity by *S. lacticus*, cultures of the associated organism (Type A) were grown in milk for from two to four days, the milk heated to kill the organisms, and *S. lacticus* added; the volatile acidity was then determined after suitable incubation. The heating consisted of standing the milk cultures in water, raising the temperature rapidly to 60° C. and then maintaining the temperature of the water between 60° and 65° C. for 45 minutes. Inoculations showed that cultures so treated contained no living organisms. For comparison, the volatile acidity produced by the associated organisms alone in unkilled cultures during the entire period of incubation was also determined. The results obtained are given in table VIII; the data show that when the associated organism was destroyed by

TABLE VIII. VOLATILE ACID PRODUCTION SECURED WHEN *S. LACTICUS* WAS ADDED TO KILLED CULTURES OF THE ASSOCIATED ORGANISM (TYPE A) ROOM TEMPERATURE INCUBATION

Inoculation	Date of Inoc. Assoc. Org.	Date of heating and of adding *S. lacticus*	Date Examined	Total acidity in percent	Volatile acidity
Assoc. Org. 1 plus *S. lacticus* A	5/13	5/15	5/19	.95	19.0
Assoc. Org. 1 plus *S. lacticus* A	5/13	5/17	5/21	.99	24.0
Assoc. Org. 1 alone without heating	5/13		5/21	.24	21.6
Assoc. Org. 2 plus *S. lacticus* B	5/13	5/15	5/19	1.03	15.6
Assoc. Org. 2 plus *S. lacticus* B	5/13	5/17	5/21	1.04	25.9
Assoc. Org. 2 alone without heating	5/13		5/21	.24	18.6
Assoc. Org. 3 plus *S. lacticus* C	5/13	5/15	5/20	.80	21.9
Assoc. Org. 3 plus *S. lacticus* C	5/13	5/17	5/21	.78	22.2
Assoc. Org. 2 alone without heating	5/13		5/21	.26	20.7

heat after being given an opportunity to grow for two to four days before the addition of *S. lacticus,* a volatile acidity (from 15.6 to 25.9) was secured which was quite in agreement with that obtained from the associated organism alone (from 18.6 to 21.6) in unkilled cultures. These results indicate that the thermostable products of the associated organism do not stimulate *S. lacticus* to the production of a high volatile acidity.

In table IX are presented data obtained when *S. lacticus* was inoculated first and the associated organism (Type A) at a later date. The results show that when *S. lacticus* was given any considerable start over the associated organism, only a low volatile acidity (from 4.2 to 10.2) was obtained, which is essentially the same as the volatile acidity secured from *S. lacticus* alone. Since the associated organism produces only a slight amount of acid in milk, it would not be expected to grow in the presence of considerable amounts of acid, altho its presence in well-ripened starter indicates that it can live under such conditions.

When *S. lacticus* was inoculated, killed with heat by the method used with the associated organism, after only a small

TABLE IX. VOLATILE ACID PRODUCTION SECURED WHEN *S. LACTICUS* WAS ADDED FIRST AND THE ASSOCIATED ORGANISM (TYPE A) LATER ROOM TEMPERATURE INCUBATION

Inoculation	Date of Inoc. *S. lacticus*	Date of Inoc. Assoc. Org.	Date Examined	Total acidity in percent	Volatile acidity
S. lacticus A plus Assoc. Org. 1	5/2	5/3	5/8	.88	7.1
S. lacticus A plus Assoc. Org. 1	5/2	5/5	5/12	.84	7.9
S. lacticus D plus Assoc. Org. 4	5/2	5/5	5/8	.84	10.0
S. lacticus A plus Assoc. Org. 1	5/27	5/28	6/3	.81	6.5
S. lacticus B plus Assoc. Org. 2	5/27	5/28	6/4	.89	4.2
S. lacticus C plus Assoc. Org. 3	6/19	6/20	6/24	.87	8.8
S. lacticus D plus Assoc. Org. 4	7/1	7/2	7/7	.89	10.2
S. lacticus D plus Assoc. Org. 4	7/1	7/3	7/7	.85	9.5

TABLE X. VOLATILE ACID PRODUCTION SECURED WHEN THE ASSOCIATED ORGANISM (TYPE A) WAS ADDED TO KILLED CULTURES OF *S. LACTICUS*
ROOM TEMPERATURE INCUBATION

Inoculation	Date of Inoc. *S. lacticus*	Date of heating and of adding Assoc. Org.	Condition of milk before heating	Date examined	Acidity at once after heating in percent	Volatile Acidity
S. lacticus A and after heating, the Assoc. Org. 1	5/27	5/28	Slight curd	6/3	.54	35.7
S. lacticus A and after heating, the Assoc. Org. 1	5/27	5/29	Solid curd	6/4		6.2
S. lacticus B and after heating, the Assoc. Org. 2	5/27	5/28	Not curdled	6/4	.29	32.8
S. lacticus B and after heating, the Assoc. Org. 2	5/27	5/29	Solid curd	6/4		4.5
S. lacticus C and after heating, the Assoc. Org. 3	6/19	6/20		6/24	.67	12.3

amount of acid had developed, and then the associated organism (Type A) added, a high volatile acidity (32.8 and 35.7) was secured. The data on this point are presented in table X. This table also gives the results of two trials in which sufficient acid to curdle the milk had developed before heating; under these conditions only a low volatile acidity (4.5 and 6.2) was secured, which was probably due to the action of *S. lacticus*.

It is entirely possible to inoculate the associated organism (Type A) later than *S. lacticus* and secure a high volatile acidity (34.2 and 40.0), as is shown by the data presented in table XI. In these trials there was one day's difference in the time of inoculation of the two organisms. It should be noted that at the time of inoculating the associated organism, there was no evidence of a change in the milk as a result of the growth of *S. lacticus*.

The data presented in tables VII to XI inclusive admit of certain conclusions as follows:

TABLE XI. VOLATILE ACID PRODUCTION SECURED WHEN THE ASSOCIATED ORGANISM (TYPE A) WAS ADDED LATER THAN *S. LACTICUS* BUT INTO MILK SHOWING NO CHANGE
ROOM TEMPERATURE INCUBATION

Inoculation	Date of Inoc. *S. lacticus*	Date of Inoc. Assoc. Org.	Condition of milk just before adding Assoc. Org.	Date examined	Total acidity in percent	Volatile acidity
S. lacticus C then Assoc. Org. 3	5/2	5/3	No change	5/8	.92	34.2
S. lacticus D then Assoc. Org. 4	5/2	5/3	No change	5/8	.91	40.0

(1) The associated organism (Type A) can be inoculated before *S. lacticus* and a high volatile acidity obtained.

(2) Where the associated organism (Type A) is destroyed with heat after a considerable period of growth and *S. lacticus* then inoculated, a volatile acidity is obtained which is essentially like that produced by the associated organism alone.

(3) If *S. lacticus* develops considerable acid before the associated organism (Type A) is inoculated, only a low volatile acidity (essentially like that secured with *S. lacticus* alone) is obtained; this occurs both when *S. lacticus* is destroyed with heat before adding the associated organism and when it is not, and is presumably due to the fact that the associated organism is unable to grow in the presence of considerable quantities of acid.

(4) By adding the associated organism (Type A) to milk in which *S. lacticus* has been destroyed by heat after the production of only a small amount of acid, a high volatile acidity is secured. It is also possible to secure a high volatile acidity by adding the associated organism to milk into which *S. lacticus* was inoculated some little time previously, provided the acid production has not gone too far.

The above conclusions suggest that a definite but not excessive amount of lactic acid and living associated organisms (Type A) constitute the combination essential to the production of a high volatile acidity. Inasmuch as this suggestion readily admits of a test by the use of sterile lactic acid and the associated organism, trials were carried out by adding sterile lactic acid to sterile milk and then inoculating the organism in question. Lactic acid from different commercial preparations was used after sterilization in the autoclave and no variation in the results obtained noted. The data secured in the preliminary trials are given in table XII.

The results given in table XII show that in the presence of a small amount of sterile lactic acid, the associated organism (Type A) produced a volatile acidity (31.2 and 31.5) higher than in milk without added lactic acid, and essentially the same as

TABLE XII. VOLATILE ACID PRODUCTION SECURED WHEN THE ASSOCIATED ORGANISM (TYPE A) WAS INOCULATED INTO MILK TO WHICH STERILE LACTIC ACID HAD BEEN ADDED

ROOM TEMPERATURE INCUBATION

Added to the 325 cc. of milk	Days held	Total acidity in percent	Volatile acidity
Associated Organism 1 alone	5	.24	22.0
Associated Org. 1 plus 1 cc. sterile lactic acid	5	.47	31.2
Associated Organism 3 alone	6	.24	22.0
Associated Org. 3 plus 1 cc. sterile lactic acid	6	.53	31.5
Uninoculated milk plus 1 cc. sterile lactic acid	7	.42	4.0

TABLE XIII. VOLATILE ACID PRODUCTION SECURED WHEN THE ASSOCIATED ORGANISM (TYPE A) WAS INOCULATED INTO MILK TO WHICH STERILE LACTIC ACID HAD BEEN ADDED

INCUBATION AT 22° C.

Added to the 325 cc. of milk	Days held	Total acidity in percent	Volatile acidity
Associated Organism 3 alone	5	.27	21.0
Associated Org. 3 plus 1 cc. sterile lactic acid	5	.53	36.5
Associated Organism 4 alone	5	.27	21.1
Associated Org. 4 plus 1 cc. sterile lactic acid	5	.52	31.0
Associated Organism 5 alone	5	.28	22.0
Associated Org. 5 plus 1 cc. sterile lactic acid	5	.50	30.9
Uninoculated milk alone	6	.19	2.0
Uninoculated milk plus 1 cc. sterile lactic acid	6	.45	4.2

that produced when the associated organism was grown in combination with *S. lacticus*.

The data given in table XIII were secured with the idea of testing out other cultures of the associated organisms and also of comparing the volatile acidity secured from uninoculated sterile milk with that secured from uninoculated sterile milk to which sterile lactic acid had been added. The results presented confirm those given in table XII, in that the volatile acid production of the associated organisms was materially increased by adding sterile lactic acid to the milk before the growth of the associated type. Table XIII also shows that the volatile acidity secured from uninoculated sterile milk to which sterile lactic acid had been added was slightly greater than that secured from the uninoculated sterile milk alone. Altho the difference is very small, there should be some explanation for it; the most logical one is that traces of lactic acid pass over in the steam distillation.

Table XIV presents data showing the volatile acid production secured in milk to which sterile lactic acid, varying in amounts from 0.25 to 2.0 cc. per 325 cc. of milk, had been

TABLE XIV. VOLATILE ACID PRODUCTION SECURED WHEN THE ASSOCIATED ORGANISM (TYPE A) WAS INOCULATED INTO MILK TO WHICH STERILE LACTIC ACID HAD BEEN ADDED IN VARYING AMOUNTS

ROOM TEMPERATURE INCUBATION

Added to the 325 cc. of milk	Days held	Total acidity in percent	Volatile acidity
Associated Organism 1 alone	6	.23	17.7
Assoc. Org. 1 plus .25 cc. sterile lactic acid	6	.36	28.1
Assoc. Org. 1 plus .5 cc. sterile lactic acid	6	.38	27.1
Assoc. Org. 1 plus 1 cc. sterile lactic acid	6	.69	31.5
Assoc. Org. 1 plus 2 cc. sterile lactic acid	6	.67	36.0
Assoc. Org. 4 plus .25 cc. sterile lactic acid	7	.42	33.6
Assoc. Org. 4 plus .5 cc. sterile lactic acid	7	.41	35.6
Assoc. Org. 4 plus 1 cc. sterile lactic acid	7	.50	30.1
Assoc. Org. 4 plus 2 cc. sterile lactic acid	7	.63	31.5
Associated Organism 6 alone	6	.23	21.0
Assoc. Org. 6 plus .25 cc. sterile lactic acid	6	.32	28.5
Assoc. Org. 6 plus .5 cc. sterile lactic acid	6	.38	31.3
Assoc. Org. 6 plus 1 cc. sterile lactic acid	6	.52	31.0
Assoc. Org. 6 plus 2 cc. sterile lactic acid	6	.59	34.5

added. The results show that the increase in volatile acid production was not proportional to the quantity of lactic acid added, even with these small amounts, and that 0.25 cc. per 325 cc. of milk gave almost as much of an increase as the larger additions.

In order to determine whether or not considerable amounts of sterile lactic acid would prevent the production of a high volatile acidity by the associated organism (Type A) the data presented in table XV were secured. These results show that, as would be expected, excessive amounts of sterile lactic acid added to milk prevent the production of volatile acid by the associated organism. Four cc. of acid per 325 cc. of milk prevented any appreciable development of volatile acidity, while with 2 cc. the usual development occurred in one case and in another only a partial development. Since the data reported in table XIV show a consistent production of a high volatile acidity when 2 cc. of lactic acid were added per 325 cc. of milk, it seems that this amount of acid can only occasionally limit the volatile acid production by associated organisms. Undoubtedly certain of these organisms may be more sensitive to acid than others; again, it is extremely difficult to distribute the acid uniformly, and an uneven distribution may allow the growth of the associated organisms by leaving certain portions of the milk with comparatively little acid.

The results already presented show that the associated organisms yield an increased amount of volatile acid in milk in the presence of lactic acid, either when produced by *S. lacticus* or when added in a sterile condition. The data reported in table XVI were obtained with the idea of finding out whether or not the addition of sterile lactates had the same effect as the addition of sterile lactic acid. Ammonium and calcium lactate, both commercial preparations, were used; the former was ster-

TABLE XV. VOLATILE ACID PRODUCTION SECURED WHEN THE ASSOCIATED ORGANISM (TYPE A) WAS INOCULATED INTO MILK TO WHICH STERILE LACTIC ACID HAD BEEN ADDED IN VARYING AMOUNTS

ROOM TEMPERATURE INCUBATION

Series No.	Added to the 325 cc. of milk	Days held	Total acidity in percent	Volatile acidity
1	Assoc. Org. 1 alone	5	.27	21.0
	Assoc. Org. 1 plus 1 cc. sterile lactic acid	5	.52	34.8
	Assoc. Org. 1 plus 4 cc. sterile lactic acid	5	1.12	5.8
2	Assoc. Org. 1 plus 1 cc. sterile lactic acid	4		32.9
	Assoc. Org. 1 plus 2 cc. sterile lactic acid	4		18.5
	Assoc. Org. 1 plus 4 cc. sterile lactic acid	4		4.5
	Assoc. Org. 2 plus 1 cc. sterile lactic acid	4		29.2
	Assoc. Org. 2 plus 2 cc. sterile lactic acid	4		30.1
	Assoc. Org. 2 plus 4 cc. sterile lactic acid	4		4.9
3	Uninoculated milk plus 1 cc. sterile lactic acid			2.5
	Uninoculated milk plus 2 cc. sterile lactic acid			2.7
	Uninoculated milk plus 4 cc. sterile lactic acid			3.7

TABLE XVI. VOLATILE ACID PRODUCTION SECURED WHEN THE ASSOCIATED ORGANISM (TYPE A) WAS GROWN IN MILK TO WHICH STERILE LACTATES HAD BEEN ADDED. AMMONIUM LACTATE ADDED AT RATE OF 1 CC. PER 325 CC. OF MILK. CALCIUM LACTATE ADDED AT RATE OF 1.25 GRAM PER 325 CC. OF MILK

ROOM TEMPERATURE INCUBATION

Organism used	Days held	Total acidity in percent	Volatile acidity
Uninoculated milk alone		.18	1.6
Uninoculated milk plus ammonium lactate		.18	1.5
Assoc. Org. 1 plus ammonium lactate	4	.28	19.6
Assoc. Org. 2 plus ammonium lactate	5	.34	20.2
Assoc. Org. 8 plus ammonium lactate	7	.28	15.2
Assoc. Org. 9 plus ammonium lactate	7	.26	17.2
Uninoculated milk alone			2.4
Uninoculated milk plus calcium lactate		.28	3.2
Assoc. Org. 8 alone	7	.27	19.9
Assoc. Org. 9 alone	7	.20	19.0
Assoc. Org. 8 plus calcium lactate	7	.29	19.0
Assoc. Org. 9 plus calcium lactate	7	.30	18.0

ilized in the autoclave and added at the rate of 1 cc. per 325 cc. of milk, while with the latter, a strong aqueous solution was made, sterilized and added at such a rate that 1.25 grams of the salt were employed per 325 cc. of milk.

The results given in table XVI indicate that sterile lactates did not yield volatile acid when they were added to milk in which the associated organism was grown; the volatile acidities obtained were essentially the same as those secured in milk without any additions. Suzuki, Hastings and Hart (31) concluded from a study of the production of volatile fatty acids and esters in cheddar cheese that the principal source of acetic and propionic acids was probably lactates. They used calcium lactate and inoculated bits of cheese instead of specific organisms. Apparently, lactates are not attacked by the associated organisms in the way they are by the mixed flora found in cheese.

An analysis of the data given in tables VII to XV, inclusive, indicates that the greater volatile acid production secured with starters than with pure cultures of either the associated organism (Type A) or *S. lacticus*, is due to the associated organism acting on the lactic acid produced by *S. lacticus*. The combination necessary for a high volatile acid production is, as has already been pointed out, a living associated organism and lactic acid in the proper amounts.

THE SOURCE OF THE VOLATILE ACIDITY PRODUCED IN MILK BY THE ASSOCIATED ORGANISM (TYPE A) ALONE

Altho the presence of lactic acid in milk enables the associated organism to produce a volatile acidity essentially the same as that produced by a good starter, this organism produces a con-

siderable amount of volatile acid in milk alone and this must come from some other material, since lactic acid is not present in milk except as produced by fermentation.

Citric acid is one of the normal constituents of milk that is present in only small amounts (about 0.1 to 0.2 percent). Bosworth and Prucha (2) found that "during the spontaneous souring of milk the citric acid in it was changed into acetic acid and carbon dioxide" and that "starter and buttermilk contained some agent capable of bringing about this change." These investigators tried a number of the common dairy bacteria, but *Bact. aerogenes* was the only one found to have the power of fermenting citric acid. Hammer and Bailey (17), however, failed to get a volatile acidity comparable with the volatile acidity produced by a starter when this organism was inoculated into milk.

In view of the results of Bosworth and Prucha, experiments were carried out to determine whether or not the associated organism (Type A) could produce volatile acid from citric acid. These trials consisted of adding to the milk in varying amounts a sterile citric acid solution of a known strength and then inoculating the associated organism. The results secured are presented in table XVII.

The data given show that the addition of sterile citric acid to milk increased very materially the volatile acidity produced by the associated organism. The addition of 0.4 gram of citric acid to 325 cc. of milk (about 0.12 percent) almost doubled the amount of volatile acidity produced; adding 0.8 gram (about 0.25 percent) increased still further the amount of volatile acid-

TABLE XVII. VOLATILE ACID PRODUCTION SECURED WHEN THE ASSOCIATED ORGANISM (TYPE A) WAS INOCULATED INTO MILK TO WHICH CITRIC ACID HAD BEEN ADDED

ROOM TEMPERATURE INCUBATION

Inoculation	Citric acid added to 325 cc. of milk	Days held	Volatile acidity
None	0.0 gram		1.8
None	.4 gram		1.4
None	.8 gram		1.5
None	1.2 gram		1.4
Associated Organism 1	0.0 gram	3	21.2
Associated Organism 1	.4 gram	3	40.0
Associated Organism 1	.8 gram	3	56.1
Associated Organism 1	1.2 gram	3	55.3
Associated Organism 3	0.0 gram	4	24.0
Associated Organism 3	.4 gram	4	42.3
Associated Organism 3	.8 gram	4	61.3
Associated Organism 3	1.2 gram	4	54.2
Associated Organism 5	0.0 gram	4	24.0
Associated Organism 5	.4 gram	4	45.6
Associated Organism 5	.8 gram	4	62.0
Associated Organism 5	1.2 gram	4	65.8

ity; while adding 1.2 grams (about 0.37 percent) gave values not very different than those secured with 0.8 gram, altho in two out of the three comparisons, the values were actually lower for the larger amount of acid. It would not be expected that the volatile acid production could be indefinitely increased by the addition of citric acid, as there must be a limit of toleration of volatile acidity for the associated organism.

Since citric acid is a normal constituent of milk and since the associated organism can produce volatile acid from this, it seems justifiable to conclude that the citric acid is the material in milk from which the volatile acid is produced by the associated organism (Type A) in pure cultures. The results secured also indicate that the agent in starter and buttermilk which Bosworth and Prucha (2) found was capable of attacking citric acid, was the associated organism.

INVESTIGATIONS WITH TYPE B.

THE VOLATILE ACID PRODUCTION OF TYPE B ALONE AND IN COMBINATION WITH *S. LACTICUS*

The statement has already been made that Type B produces large amounts of volatile acid, but results showing this have not been given. Table XVIII presents data illustrating the volatile acid production of Type B alone and in combination with *S. lacticus*; the volatile acid production of some of the *S. lacticus* cultures is also given.

TABLE XVIII. THE VOLATILE ACID PRODUCTION SECURED WITH ASSOCIATED ORGANISM (TYPE B) ALONE AND IN COMBINATION WITH *S. LACTICUS*

ROOM TEMPERATURE INCUBATION

Inoculation		Days held	Total acidity in percent	Volatile acidity
Type B	*S. lacticus*			
Assoc. Org. 101 alone		5	.72	30.8
Assoc. Org. 101	L	5	1.20	36.0
Assoc. Org. 102 alone		5	.77	36.9
Assoc. Org. 102	M	5	1.16	36.9
Assoc. Org. 103 alone		6	.68	31.0
Assoc. Org. 104 alone		6	.63	28.5
Assoc. Org. 104	N	6	1.15	39.6
	N alone	6	.83	7.5
Assoc. Org. 105 alone		6	.59	29.0
Assoc. Org. 106 alone		7	.49	32.8
Assoc. Org. 106	O	6	1.11	35.0
	O alone	6	.95	8.1
Assoc. Org. 107 alone		7	.49	36.1
Assoc. Org. 107	P	6	1.15	42.0
	P alone	6	.99	8.0
Assoc. Org. 108 alone		7	.56	34.6
Assoc. Org. 108	R (2 days later)	7	1.23	45.7
Assoc. Org. 109 alone		5	.45	23.8
Assoc. Org. 109	R (2 days later)	5	.96	38.5
Assoc. Org. 110 alone		6	.39	29.5
Assoc. Org. 110	R	6	1.14	39.1
Assoc. Org. 111 alone		6	.52	37.3
Assoc. Org. 111	R	6	1.14	40.0
	R alone	6	.90	8.0

In the data given the volatile acid production of Type B alone varies from 23.8 to 37.3 and the total acidity from .39 to .77 percent. Altho the results show considerable variation, the volatile acidity and total acidity produced are in general higher than those produced by Type A; usually the volatile acidity produced by Type B when grown alone is about as high as that produced by a good starter.

When Type B was grown in combination with *S. lacticus,* the volatile acidity produced varied, in the data given in table XVIII, from 35.0 to 45.7 and in general was higher than the volatile acidity secured with Type B alone, altho in one instance the value for the combination (36.9) was the same as the value for Type B alone. The volatile acidities of the *S. lacticus* cultures for which results are given, varied from 7.5 to 8.1, and these results are sufficiently representative for this organism so that they can be used for all of the *S. lacticus* cultures employed in table XVIII. With a number of the comparisons, the addition of the volatile acidities for the two organisms gives a value greater than the volatile acidity produced by the two in combination; the adding of the values for the two organisms introduces an error in that the initial volatile acidity of the milk is included twice, but this is relatively unimportant. It seems, accordingly, that, while there was a somewhat greater volatile acidity secured when Type B was combined with *S. lacticus* than when it was grown alone, this was often accounted for by the volatile acid production of *S. lacticus.*

ISOLATION OF THE ASSOCIATED ORGANISM OF THE B TYPE

The associated organism of the B type was isolated by the plate method in all cases. When colonies are picked from whey agar plates poured from starters, the B type is quite readily detected because of its reaction in litmus milk. This consists in young cultures of a definite reddening without coagulation, except for a very small amount of curd in the bottom of the tubes in a few instances; with old cultures coagulation was occasionally complete. Reduction of the litmus was never observed except in the small coagulated portions and in these instances is probably to be explained by the rapid penetration of the oxygen of the air as long as the milk is uncoagulated. In the old coagulated cultures reduction did not occur.

In using the method of repeated transfers Type B was never isolated, altho this organism was present in some of the starters with which the method was employed.

Testing out the organism secured. The associated organism of the B type was identified by its reaction in milk as outlined above and by its volatile acid production. The volatile acidity to be expected is shown in table XVIII and, while there is con-

siderable variation between different cultures, the volatile acidities are always much in excess of the results secured with *S. lacticus.*

PRESENCE OF TYPE B IN STARTERS, BOTH CREAMERY AND COMMERCIAL

The associated organism of the B type was not found so constantly in the starters examined, either creamery or commercial, as was the A type. In one of the commercial starters it was found very regularly, while it has been occasionally found in others. It has also been found in natural starters in a number of instances. Some very good starters have never yielded Type B in a considerable number of examinations, and only the one commercial culture yielded it regularly.

THE SOURCE OF THE VOLATILE ACIDITY PRODUCED BY TYPE B IN PURE AND MIXED CULTURES

With Type B, the difference between the volatile acidity produced in pure culture and that produced in combination with *S. lacticus* is in general less than with Type A; accordingly, the important consideration with Type B is the source of the volatile acidity in pure cultures.

Table XIX presents data showing the effect of adding citric acid on the volatile acidity produced by Type B in milk; from these results it is evident that the addition of citric acid to milk greatly increased the volatile acid production of the organism (1.5 grams of citric acid to 325 cc. of milk yielding volatile acidities from 67.6 to 87.1) and they accordingly indicate that the citric acid normally present in milk is at least one of the sources of the volatile acidity produced by Type B.

Data showing the influence of adding sterile lactic acid or lactates to milk on the volatile acid production of Type B are presented in table XX. Milk to which lactic acid had been added

TABLE XIX. VOLATILE ACID PRODUCTION SECURED WHEN THE ASSOCIATED ORGANISM (TYPE B) WAS INOCULATED INTO MILK TO WHICH CITRIC ACID HAD BEEN ADDED

ROOM TEMPERATURE INCUBATION

Inoculation	Citric acid added to 325 cc. of milk	Days held	Volatile acidity
None	0.0 gram		2.6
None	1.5 gram		2.7
Associated Organism 113	0.0 gram	7	29.3
Associated Organism 113	1.5 gram	7	82.9
Associated Organism 114	0.0 gram	8	27.4
Associated Organism 114	1.5 gram	8	87.1
Associated Organism 110	0.0 gram	9	32.4
Associated Organism 110	1.5 gram	8	83.2
Associated Organism 111	0.0 gram	7	30.2
Associated Organism 111	.75 gram	8	52.6
Associated Organism 111	1.5 gram	7	79.2
Associated Organism 112	0.0 gram	8	18.5
Associated Organism 112	1.5 gram	8	67.6

TABLE XX. VOLATILE ACID PRODUCTION SECURED WHEN THE ASSOCIATED ORGANISM (TYPE B) WAS GROWN IN MILK TO WHICH STERILE LACTIC ACID OR LACTATES HAD BEEN ADDED

Lactic Acid Added at Rate of 1 cc. per 325 cc. of Milk.
Ammonium Lactate Added at Rate of 1 cc. per 325 cc. of Milk.
Calcium Lactate Added at Rate of 1 gram per 325 cc. of Milk.

ROOM TEMPERATURE INCUBATION

Added to the milk	Days held	Volatile acidity
Uninoculated milk alone		2.6
Uninoculated milk plus lactic acid		5.7
Uninoculated milk plus ammonium lcatate		1.0
Uninoculated milk plus calcium lactate		1.6
Associated Organism 113 alone	7	29.3
Associated Organism 113 plus lactic acid	7	33.8
Associated Organism 113 plus ammonium lactate	7	20.2
Associated Organism 113 plus calcium lactate	7	27.7
Associated Organism 114 alone	8	27.4
Associated Organism 114 plus lactic acid	8	38.3
Associated Organism 114 plus ammonium lactate	8	18.8
Associated Organism 114 plus calcium lactate	8	34.3
Associated Organism 110 alone	8	32.4
Associated Organism 110 plus lactic acid	8	38.5
Associated Organism 110 plus ammonium lactate	9	12.6
Associated Organism 110 plus calcium lactate	9	28.6
Associated Organism 111 alone	7	30.2
Associated Organism 111 plus lactic acid	7	33.5
Associated Organism 111 plus ammonium lactate	8	26.7
Associated Organism 111 plus calcium lactate	7	27.5
Associated Organism 112 alone	8	18.5
Associated Organism 112 plus lactic acid	8	30.7
Associated Organism 112 plus ammonium lactate	8	13.1
Associated Organism 112 plus calcium lactate	8	15.8

gave in general a somewhat higher volatile acidity than milk alone, and the difference was greater when the volatile acidity of Type B was low than when it was high. When the volatile acidity of the uninoculated milk to which sterile lactic acid had been added is taken into consideration, the results secured are in agreement with those obtained when Type B was grown in combination with *S. lacticus*. The addition of lactates usually resulted in a decreased volatile acid production and the decrease was greater with ammonium lactate than with calcium lactate.

From the standpoint of volatile acid production, the situation with Type B is somewhat different than with Type A.

Altho both organisms produce volatile acid from citric acid, the volatile acid production in pure culture with Type B is considerably higher in general than with TypeA. The volatile acid production of Type A is definitely increased by the addition to the milk of lactic acid, either in a sterile condition or as a result of fermentation, while this is not regularly the case with Type B. On the other hand Type B produces considerable acid in the milk, while Type A does not. This suggests that Type B produces volatile acid either directly or indirectly from some constituent of the milk other than citric acid. It is certain that only a small part of the acid produced in milk by Type B is volatile and unreported data that have been obtained

show that at least some of the non-volatile acid is lactic. Accordingly it may be that while Type A takes the lactic acid elaborated by *S. lacticus* and changes it into volatile acid, Type B produces its own lactic acid to be used in such a transformation. This explanation accounts for the fact that Type B often produces about as much volatile acid when grown alone as when grown in combination with *S. lacticus,* the volatile acidity produced by *S. lacticus* being deducted from the value for the mixture of organisms.

With both Type A and Type B a much higher volatile acidity was secured by adding citric acid to milk than in any other way. This suggests that citric acid is, under natural conditions, a more satisfactory and efficient source of volatile acid than anything else. Type A and probably also Type B, produce comparatively little volatile acid from other sources, and while this is sufficient to raise the volatile acid production somewhat above that coming from the citric acid alone, it explains the limit of from 30 to 40 for the volatile acidity of starters under ordinary conditions; with the amounts of lactic acid in starters the volatile acidity would undoubtedly be much above these values if lactic acid were an entirely satisfactory source of volatile acid.

STUDIES OF THE ASSOCIATED ORGANISMS WHICH DEAL WITH BOTH TYPE A AND TYPE B.

Some of the results obtained in the study of the associated organisms are such that they can best be considered by dealing with the two types at the same time.

THE INHIBITORY INFLUENCE OF THE ASSOCIATED ORGANISMS ON THE ACID DEVELOPMENT OF *S. LACTICUS*

In the combining of *S. lacticus* and the associated organisms, Type A was shown to be prevented from producing the usual volatile acidity by excessive amounts of acid and it is probable that the activity of Type B could be limited in the same way. The associated organisms, however, also restrain the activity of *S. lacticus,* as is evident from the following consideration.

In preparing mixtures of *S. lacticus* and the associated organisms for use in developing starters, it was the usual practice to inoculate the associated organism into sterile milk on one day and *S. lacticus* on the next, so that the associated organism would have a good opportunity for growth. It was observed that the milk in which one of the associated types had grown for a day before *S. lacticus* was inoculated did not coagulate as rapidly as did the milk containing *S. lacticus* alone. Altho equal amounts of *S. lacticus* had not been inoculated in the dif-

ferent lots of milk, the very great difference in the time of coagulation suggested that the associated organism delayed the acid development by *S. lacticus*. This suggestion was tested out in a series of experiments in which equal amounts of a suspension of *S. lacticus* were used for inoculating the milk with and without the associated organism, *S. lacticus* being inoculated one day after the associated type. A portion of the data obtained, sufficient to show the general trend of the results, is presented in table XXI. The tests were then duplicated, except that the two organisms were inoculated at the same time. The results secured are given in table XXII.

From tables XXI and XXII it is evident that the associated organisms delay the development of acid by *S. lacticus*. This is evident in several ways.

First,—in the time required for coagulation. In general the milk containing only *S. lacticus* curdled before milk of the same lot inoculated with an equal amount of a suspension of *S. lacticus*, but also containing the associated organism. The extent to which the coagulation was delayed varied greatly due prob-

TABLE XXI. INFLUENCE OF THE ASSOCIATED ORGANISMS ON THE ACID DEVELOPMENT OF *S. LACTICUS*

Associated Organisms Inoculated 1 day before *S. lacticus*

ROOM TEMPERATURE INCUBATION

Series No.	Assoc. Org.		Assoc. Org. Inoc.	*S. lacticus* Inoc.	Curdling began after approx.	Curdling complete after approx.	Acidity in per cent on date indicated	Acidity in per cent on date indicated	Acidity in per cent on date indicated
	No.	Type							
1							On 1-10	On 1-11	On 1-12
	10	A	1-7	1-8	23 hr.	28 hr.	.87	.99	1.10
	11	A	1-7	1-8	24 hr.	30 hr.	.87	1.02	1.08
	12	A	1-7	1-8	24 hr.	30 hr.	.87	1.03	1.07
	None			1-8	18 hr.	18.5 hr.	.92	1.03	1.09
							On 1-11	On 1-12	On 1-15
2	13	A	1-8	1-9		42 hr.	.93	.99	1.10
	117	B	1-8	1-9			.37	.45	.97
	112	B	1-8	1-9	23 hr.	42 hr.	.90	1.00	1.13
	None			1-9	16 hr.	18 hr.	.97	1.03	1.09
							On 1-14	On 1-16	
3	12	A	1-11	1-12	26 hr.	39 hr.	.86	.95	
	10	A	1-11	1-12	26 hr.	39 hr.	.86	.95	
	112	B	1-11	1-12		39 hr.	.90	1.02	
	117	B	1-11	1-12		39 hr.	.85	.99	
	None			1-12	21 hr.	23 hr.	.86	.92	
	None			1-12	21 hr.	23 hr.	.85	.92	
							On 1-21		
4	14	A	1-18	1-19	39 hr.		.58		
	15	A	1-18	1-19	39 hr.		.54		
	110	B	1-18	1-19			.34		
	112	B	1-18	1-19	39 hr.		.61		
	None			1-19		25 hr.	.83		
	None			1-19		25 hr.	.81		
5	12	A	1-26	1-27	48 hr.				
	16	A	1-26	1-27					
	116	B	1-26	1-27	40 hr.				
	111	B	1-26	1-27	48 hr.				
	None			1-27		25 hr.			
	None			1-27		25 hr.			

TABLE XXII. INFLUENCE OF THE ASSOCIATED ORGANISMS ON THE ACID DEVELOPMENT OF *S. LACTICUS*

Associated Organisms and *S. lacticus* Inoculated at the same time

ROOM TEMPERATURE INCUBATION

Series No.	Assoc. Org.		Time of Inoc.	Curdling began after approx.	Curdling complete after approx.	Acidity in percent on date indicated	Acidity in percent on date indicated
	No.	Type					
						On 1-14	On 1-19
1	12	A	1-13		15 hr.	.85	1.19
	17	A	1-13	21 hr.	24 hr.		1.19
	112	B	1-13	15 hr.	19 hr.		1.21
	120	B	1-13		15 hr.	.84	1.10
	None		1-13		15 hr.	.81	1.16
	None		1-13		15 hr.	.81	1.16
						On 1-15	On 1-19
2	10	A	1-14		39 hr.		1.12
	17	A	1-14		39 hr.		1.13
	110	B	1-14	24 hr.	27 hr.	.66	1.14
	112	B	1-14	23 hr.	27 hr.	.65	1.23
	18	A	1-14		39 hr.		1.13
	None		1-14	23 hr.	24 hr.	.67	1.12
						On 1-21	On 1-27
3	10	A	1-19		39 hr.	.80	1.01
	19	A	1-19		39 hr.	.79	1.01
	110	B	1-19	39 hr.		.63	.99
	112	B	1-19	25 hr.		.79	1.00
	None		1-19		25 hr	.83	1.00
	None		1-19		25 hr.	.81	.99
4	12	A	1-27		40 hr.		
	16	A	1-27	40 hr.	42 hr.		
	116	B	1-27	40 hr.	42 hr.		
	111	B	1-27	42 hr.			
	None		1-27		25 hr.		
	None		1-27		25 hr.		

ably to variations in the extent of the development of the associated organisms in the different cultures.

Second,—in the time elapsing between beginning and complete coagulation. In milk containing *S. lacticus* alone, only a short time elapsed from beginning to complete coagulation [the results reported agreeing in this respect with the findings of Van Slyke and Baker (32) in their study of the souring of milk], while in milk containing *S. lacticus* together with the associated organism, a much longer time was required to effect the complete curdling of the milk after a slight coagulation was evident. The results obtained with the mixed cultures are not exactly comparable with starters, since the associated organisms undoubtedly made up a larger percentage of the total flora in the mixtures than they do in starters under ordinary conditions.

Third,—in the acidity developed. Early in the holding period, the acidity of the milk containing *S. lacticus* alone was generally higher than the acidity of milk of the same lot containing this organism in combination with one of the associated types. After a more extended holding period, however, it was common to find the acidity of the mixed cultures slightly higher than that of cultures of *S. lacticus* alone; the differences were

so small that they would not be of any significance if they were not in agreement with the results of Hammer and Bailey (17), which show quite generally a slightly greater acidity in cultures showing a high volatile acidity (that is, cultures containing the associated organism) than in pure cultures of *S. lacticus*.

Trials were also run in which the associated organism was grown in milk for one day and then killed by heat before adding *S. lacticus*. The results were not consistent, but in the majority of instances the killed cultures exerted a slight restraining action on the rate of acid development by *S. lacticus*, altho the influence was not nearly as great as the influence of the unheated cultures.

COMPARATIVE NUMBERS OF *S. LACTICUS* AND ASSOCIATED ORGANISMS IN STARTERS

The results of Hammer and Bailey (17) show that when a starter was diluted and varying amounts added to sterile milk, the tubes (showing growth) which contained the smaller amounts of starter yielded only a low volatile acidity, while those containing larger amounts gave a high volatile acidity that was essentially the same as that secured with considerable amounts of starter. This indicates that in the original material *S. lacticus* was generally present in larger numbers than was the associated organism.

More definite information on the comparative numbers of *S. lacticus* and the associated organisms was secured by pouring whey agar plates with the starter to be studied and picking off a considerable number of colonies into litmus milk after a satisfactory incubation of the plates. The tubes of litmus milk were held at 20° C. for several days and then examined and the organisms classified. The *S. lacticus* cultures were readily detected, if they were typical, by the reaction in litmus milk; the associated organism of the B type could be told in much the same way and the A type was detected by taking the tubes showing no change and staining or culturing on whey agar slopes to be sure that organisms were present. The volatile acid production of the organisms classed as the associated types could not be checked out in all cases, because of the immense amount of work involved. Previous experience with the organisms, however, and a careful study of the cultures secured, indicated that the classification of these organisms was approximately correct. The number of colonies picked varied a great deal, but contiguous colonies in sufficient numbers so that the percentages would be reasonably accurate were secured.

The results obtained varied a great deal. In a few instances plates were secured from starters in which all or nearly all of

TABLE XXIII. COMPARATIVE NUMBERS OF *S. LACTICUS* AND ASSOCIATED ORGANISMS IN CREAMERY STARTERS

CARRIED IN ONE CREAMERY

Starter from culture put out by manufacturer	Total No. colonies picked	Percent *S. lacticus*	Percent Associated Organisms	
			Type A	Type B
A	52	90	10	0
A	49	88	12	0
E	52	90	10	0
E	49	94	6	0
D	40	87.5	12.5	0
C	43	72	28	0
A	68	88	12	0
——*	92	63	2	35

* Natural starter.

the colonies belonged to one of the associated types. The reason for this was not evident from the available history of the starters or from anything that could be learned regarding them in the laboratory, and accordingly these unusual results are not included in the tables presented.

Table XXIII gives the comparative numbers of *S. lacticus* and the associated organisms in creamery starters carried in one creamery. The starters were propagated from cultures coming from a number of manufacturers. From 40 to 92 colonies were picked from the plates poured with each starter. With the eight starters for which results are given, from 63 to 94 percent of the colonies picked were of the *S. lacticus* type, leaving the percentage of the associated organisms from 6 to 37. Seven of the eight starters showed associated organisms of the A type only, while the one remaining, which was a natural starter, showed Type B to the extent of 35 percent and Type A to the extent of 2 percent of the total flora.

Additional data on the comparative numbers of *S. lacticus* and the associated organisms in creamery starters are given in table XXIV. These results were obtained on starters carried under varying conditions by different persons, all of whom had had considerable experience in starter making. The colonies picked from the plates poured with each starter varied

TABLE XXIV. COMPARATIVE NUMBERS OF *S. LACTICUS* AND ASSOCIATED ORGANISMS IN CREAMERY STARTERS

Starter from culture put out by manufacturer	Total No. colonies picked	Percent *S. lacticus*	Percent Associated Organisms	
			Type A	Type B
H	75	97	3	0
H	56	98	2	0
C	66	91	9	0
D	72	99	1	0
A	64	97	3	0
C	72	94	6	0
D	77	96	4	0
E	62	89	11	0
H	53	98	2	0
A	68	99	1	0
——*	63	87	0	13

* Natural starter.

TABLE XXV. COMPARATIVE NUMBERS OF *S. LACTICUS* AND ASSOCIATED ORGANISMS IN COMMERCIAL CULTURES DISTRIBUTED IN MILK

Culture put out by manufacturer	Total No. colonies picked	Percent *S. lacticus*	Percent Associated Organisms	
			Type A	Type B
C	96	92	8	0
C	76	32	0	68
A	73	92	8	0
A	34	97	3	0
C	37	95	0	5
C	33	88	3	9
A	38	95	5	0

from 53 to 77. In the 11 starters studied, the percentage of the *S. lacticus* type varied from 87 to 99, leaving from 1 to 13 percent of the flora made up of associated organisms. Ten of the starters had only Type A representing the associated forms, while one had only Type B, and in this the associated organisms made up a larger part of the flora than in any of the other starters.

Table XXV presents data showing the comparative numbers of *S. lacticus* and the associated organisms in commercial cultures distributed in milk. The cultures usually arrived uncoagulated and the material worked with was obtained by allowing these to stand over night at room temperature so that coagulation would occur. The products of only two manufacturers are included; these were selected because they represent very different floras, the one never containing Type B, while the other usually showed this type. From 33 to 96 colonies were picked from the plates poured with each starter and of these, 32 to 97 percent were *S. lacticus*; the associated organisms accordingly made up from 3 to 68 percent of the total flora. The product of manufacturer A in three trials showed only the A type of associated organism, while the product of manufacturer C in four trials showed Type B alone in two, Type A alone in one and both Type A and Type B in one.

Data showing the comparative numbers of *S. lacticus* and the associated organisms in powdered commercial cultures after a number of transfers in milk are presented in table XXVI. The number of colonies picked from the plates poured with the three starters representing two manufacturers varied from 35 to 61, and of these from 81 to 89 percent were *S. lacticus*, leaving from

TABLE XXVI. COMPARATIVE NUMBERS OF *S. LACTICUS* AND ASSOCIATED ORGANISMS IN STARTERS MADE BY CARRYING POWDERED COMMERCIAL CULTURES IN STERILE MILK

Culture put out by manufacturer	Transfer in sterile Milk	Total No. colonies picked	Percent *S. lacticus*	Percent Associated Organisms	
				Type A	Type B
A	6	35	86	14	0
G	5	61	89	11	0
G	7	27	81	19	0

11 to 19 percent of the flora made up of associated organisms. Type A was the only associated form found.

From the data presented in tables XXIII to XXVI inclusive, it is evident that in general both the creamery and commercial cultures studied contained much larger numbers of *S. lacticus* than of the associated organisms. *S. lacticus* often made up 90 percent or more of the total flora and only occasionally fell under 75 percent. The results also indicate that Type B is much less frequently met with than Type A, but that Type B, when present, sometimes makes up a considerable portion of the flora. Often the comparative numbers of the associated organisms were so small (1 to 3 percent) that it would seem that this type could not be of any significance, but the constant difference in the volatile acidity produced when the associated types were present or absent definitely disproves this.

In addition to the results presented, a number of starters were examined at different acidities. ages, etc., in an attempt to determine under what conditions the associated organisms were present in largest numbers compared to *S. lacticus*. With a given starter the acidity seemed to have little influence on the percent of the flora made up of associated types when the platings were made within a day or so of each other. From the fact that Type A commonly lives longer than *S. lacticus* in old acid milk, it would seem that in starter held for several days, there should be exceptionally high percentages of this form. In the trials made this was not the case, however, and the same conclusion is suggested by the results obtained with the method of isolation consisting of transferring from old acid milk, since in the successful isolations the numbers of colonies of the associated organism present on the whey agar slope were often very small. Attempts were also made to determine whether the associated organisms made up a larger percentage of the total flora at the top or bottom of a deep layer of starter, but the results obtained admitted of no definite conclusions.

THE PREPARATION OF SATISFACTORY STARTERS

The preparation of satisfactory starters was attempted by mixing *S. lacticus* with the associated organisms and carrying the mixtures in pasteurized milk in the same way that starters are ordinarily carried. The mixtures were usually prepared by inoculatng the associated type into a tube of sterile milk a day before *S. lacticus* and then holding the tube until coagulation occurred. In general the method yielded starters that were satisfactory and some of them were exceptionally good; in some few cases, however, the preparations secured were of a poor quality and were discarded after a few transfers.

Type A was used in some of the starters, Type B in some and in a few both were used. Satisfactory results were usually secured with all of these and the data obtained did not admit of any conclusions as to whether either organism or the combination was best.

Attempts were repeatedly made to prepare satisfactory starters by carrying pure cultures of *S. lacticus* in pasteurized milk. Altho such cultures were often carried thru many transfers, a satisfactory starter was never secured. The products obtained were always flat and undesirable and not at all suggestive of the pleasing odor and flavor present in a good starter.

The results obtained make it evident that the associated types are important from the standpoint of starter making and that satisfactory starters are not pure cultures of *S. lacticus*.

CHARACTERISTICS OF TYPE A AND TYPE B

The associated organisms isolated from starters were considered to represent two distinct types, mainly because of differences in the total acid development, the ability to grow at 37° C. and the volatile acidity produced. From the data that have been presented, it seems that the difference in the volatile acidity produced is dependent upon the difference in the total acidity, since some of the volatile acidity produced by Type B likely comes from the lactic acid produced by this organism.

While it is possible to classify most of the associated organisms as Type A or Type B, the results secured with some of the cultures studied suggest that they stand between the two characterized types. This indicates that the general group is more or less variable and that probably the types recognized are simply those of most frequent occurence in the starters studied.

Cultures of Type A and Type B were studied and the following descriptions prepared from the results obtained. The name *Streptococcus citrovorus* is proposed for Type A and the name *Streptococcus paracitrovorus* for Type B.

DESCRIPTION OF TYPE A. STREP. CITROVORUS

MORPHOLOGY:

Form: The organism was spherical or only slightly longer than broad in preparations made from various media.

Size: The spherical cells had a diameter of from .6 to 1.0 micron, while the cells that were slightly elongated had a length of from .7 to 1.1 microns and a breadth a very little less.

Arrangement: The organisms were generally arranged in pairs or chains. Chain formation varied with the media, the chains being much longer in liquid media than on agar. The longest chains were observed in sugar bouillons and they sometimes contained 50 or more cells. There was also considerable variation in chain formation between the different cultures.

Motility: Motility was never observed, even in young bouillon cultures.

Staining Reaction: The organism stained readily with the usual stains. The Gram stain was positive, but preparations from young whey agar cultures sometimes showed a few negative cells; the negative cells apparently became more common as the cultures increased in age, but even in old cultures the cells were largely positive.

Spore Formation: No evidence of spore formation was detected in stained preparations. Cultures of various ages and from different media failed to resist 80°C. for ten minutes.

CULTURAL CHARACTERISTICS:

Agar Streak: On whey agar, growth was usually evident after 24 hours at room temperature as a series of small, non-viscid, closely-placed colonies. With age there was an increase in the size of the colonies and sometimes in the lower portion of the culture the colonies grew together and formed a mass of growth. The colonies often had a white or dirty white color, but sometimes, especially when young, had an appearance suggesting water droplets. Very commonly in older cultures some of the colonies were considerably larger than the average and these were definitely white or dirty white. Growth on beef infusion agar was essentially the same as that on whey agar, but beef extract agar yielded only a slow growth if it occurred at all. At 37°C., growth did not occur on any of the media tried.

Agar Stab: In whey agar slight growth was evident along the line of inoculation after 24 hours at room temperature. With age there was an increase in the amount until it was definitely filiform and non-viscid, equal in amount along the entire length of the stab, but with only a very little white, smooth-edged growth at the surface. Beef infusion agar stabs gave only a little, if any growth. Growth did not occur at 37°C.

Agar Plate Colony: Whey agar plates showed growth after 24 hours at room temperature. After 48 hours the colonies were readily differentiated into small, usually round, white, non-viscid, smooth-edged, only slightly raised surface colonies and small, compact, white, round to ellipsoidal, smooth-edged sub-surface colonies. With age there was an increase in the size of the colonies, but they always remained small and only in extreme cases reached 1 mm. in diameter. The beef infusion agar plates gave essentially the same growth as whey agar, while beef extract agar plates gave little if any growth.

Whey Gelatine Stab: Growth was evident at 20°C. in 24 hours and increased with age. The young cultures looked as tho the growth was made up of closely aggregated colonies, but later there was more of a filiform appearance. The development was quite uniform thruout the length of the stab, with very little growth at the surface.

Bouillons: In plain beef extract bouillon growth did not take place. At room temperature growth usually occurred as a sediment in the presence of fructose, galactose, glucose, lactose, or maltose, but was not evident in the presence of glycerol, sucrose, mannitol, salicin, raffinose, inulin or starch.

Potato: Potato cultures showed no evidence of growth.

Dunham's Solution: There was no evidence of growth in Dunham's solution.

Uschinsky's Solution: There was no evidence of growth in Uschinsky's solution.

Plain Milk: There was no change evident in plain milk.

Litmus Milk: Litmus milk cultures showed no pronounced change. There was sometimes a slight reduction, which was more evident in faintly colored litmus milk than in heavily colored and which could

ordinarily be definitely told only when uninoculated tubes were available for comparison. Old cultures in heavily colored litmus milk showed an extremely faint, reddish tinge which could not be definitely detected without a comparison with uninoculated tubes; the cultures would not be considered as showing acid development.

Peptone (1 percent) Milk: There was no change evident except a slight coagulation in the bottom of the tubes in a few instances.

Peptone (1 percent) Litmus Milk: A reddening of the litmus was evident in from one to three days and in a few instances there was a slight coagulation in the bottom of the tubes.

BIO-CHEMICAL FEATURES:

Gas Production: Gas production was not evident in milk or bouillons containing various fermentable materials.

Acid Production: The acid production in milk was very slight, the usual acidities after long incubation at room temperature being about .25 percent calculated as lactic acid. Beef extract bouillon containing fructose, galactose, glucose, lactose, or maltose usually showed a slight increase in acidity, but with glycerol, sucrose, mannitol, salicin, raffinose, inulin or starch no increase was observed.

Oxygen Relation: The organism was facultative.

DESCRIPTION OF TYPE B. STREP. PARACITROVORUS

MORPHOLOGY:

Form: The organism was spherical or only slightly longer than broad in preparations made from various media.

Size: The spherical cells had a diameter of from .6 to 1.0 micron. The cells that were slightly elongated had a length of from .6 to 1.1 microns and a breadth only a little less.

Arrangement: The organisms were generally arranged in pairs or short chains in preparations made from various media. There was more of a tendency to chain formation in milk or sugar bouillons than on agar. Considerable variation existed in the chain-forming tendencies of different cultures.

Motility: Motility was not observed, even in young bouillon cultures.

Staining Reaction: The organisms stained readily with the ordinary stains. The Gram stain was positive, but some negative cells were usually present, especially in old cultures.

Spore Formation: Stained preparations showed no evidence of spores, and cultures of various ages and from different media failed to resist 80° C. for ten minutes.

CULTURAL CHARACTERISTICS:

Agar Streak: Growth was usually evident on whey agar after 24 hours at room temperature as a series of small, non-viscid, closely-grouped colonies. There was an increase in the size of the colonies as the cultures aged and sometimes a growing together of the colonies in the lower part of the culture until a mass of solid growth resulted. The young colonies usually had the appearance of water droplets, but later became white or cream-colored. In older cultures, some colonies considerably larger than the others were often present. On beef infusion agar, growth was essentially the same as on whey agar, but it was very slight, if it occurred at all, on beef extract agar. At 37°C. growth was essentially the same as at room temperature.

Agar Stab: In whey agar there was a slight growth suggesting a series of colonies along the line of inoculation after 24 hours at room temperature. With age there was an increase in growth which even-

tually became definitely filiform and non-viscid, about equal in amount thruout the length of the stab, and with very little surface growth. Beef infusion agar stabs had about the same appearance as those in whey agar, but beef extract yielded only very little, if any, growth. At 37°C., agar stabs gave about the same growth as at room temperature.

Agar Plate Colony: On whey agar plates growth was evident after 24 hours, and after 48 hours the colonies were readily differentiated into small, usually round, white, non-viscid, smooth-edged slightly raised surface colonies, and small, compact, white, round to ellipsoidal, smooth-edged sub-surface colonies. The colonies increased in size with age but only occasionally reached 1 mm. in diameter. Beef infusion agar plates gave the same type of growth as whey agar, but growth on beef extract agar plates was very poor, if it occurred at all.

Whey Gelatine Stab: After 24 hours at 20°C., growth was evident, and after about two days it had the appearance of a series of colonies along the line of puncture. Later the growth had a definitely filiform appearance. Development was quite uniform thruout the length of the stab, with very little growth at the surface.

Bouillons: In plain beef extract bouillon no growth was evident at either room temperature or 37°C. At either temperature growth usually was evident as a sediment in the presence of fructose, galactose, glucose, lactose, or maltose and sometimes in the presence of sucrose, but not with glycerol, mannitol, salicin, raffinose, inulin or starch.

Potato: Potato cultures showed no evidence of growth.

Dunham's Solution: Dunham's solution showed no evidence of growth.

Uschinsky's Solution: There was no evidence of growth in Uschinsky's solution.

Plain Milk: There was no change evident in plain milk, with the exception of a very slight coagulation in the bottom of the tubes with some cultures and an occasional complete coagulation in old cultures. The same results were secured at 37°C. and room temperature.

Litmus Milk: Inoculated litmus milk showed a reddening of the litmus thruout in from one to three days. Later with certain cultures there was a slight coagulation in the bottom of the tubes and occasionally complete coagulation in old cultures. There was no reduction of the litmus, except in the slight amount of curd at the bottom of certain tubes. The changes occurred at both 37°C. and room temperature.

Peptone (1 *percent*) *Milk*: Inoculated peptone (1 percent) milk showed a complete coagulation with some cultures, while with others there was only a small amount of curd in the bottom of the tubes. Often the coagulated cultures were "wheyed off" and exhibited breaks in the curd suggestive of gas holes. Development was the same at 37°C. as at room temperature.

Peptone (1 *percent*) *Litmus Milk*: Inoculated peptone (1 percent) litmus milk showed a reddening of the litmus thruout in from one to three days; with some cultures there was later complete coagulation, while with others there was only a small amount of curd in the bottom of the tubes. The coagulated cultures usually "wheyed off" and showed holes in the curd suggestive of gas production. There was ordinarily no reduction of the litmus. Development was the same at 37°C. as at room temperature.

BIO-CHEMICAL FEATURES:

Gas Production: Gas formation was not observed in beef extract bouillons containing various fermentable materials. In milk which

was coagulated after the addition of peptone small holes suggestive of gas were sometimes observed and in milk cultures heated in boiling water there seemed to be some gas driven off.

Acid Production: The acidity of fermented milk varied from about .4 to about .75 percent calculated as lactic acid. Beef extract bouillon containing fructose, galactose, glucose, lactose, maltose, or, with certain organisms, sucrose usually showed a slight increase in acidity at either 37°C. or room temperature but with glycerol, mannitol, salicin, raffinose, inulin, or starch no such increase was observed.

Oxygen Relation: The organism was facultative.

DISCUSSION OF RESULTS

The results herein reported supplement the reports of Hammer and Bailey (17), Storch (30), and Boekhout and Ott de Vries (1) which show that organisms in addition to *S. lacticus* are necessary in a good starter, by pointing out that there are at least two types of such organisms, with enough difference between them to justify their separation. It is, of course, entirely possible that further study will show that still other types exist. The importance of these forms is evident from their ability to produce a satisfactory starter when either one or a mixture of the two is grown in combination with *S. lacticus* and carried in pasteurized milk in the way that a starter is usually carried.

From the data secured it seems that Type A produces volatile acid from the citric acid normally present in milk and from the lactic acid produced by *S. lacticus*, while Type B produces volatile acid from the citric acid and probably also from the lactic acid which the organism is capable of producing in milk. The lactic acid produced by *S. lacticus*, however, is also of importance in freeing from combinations with the milk constituents the volatile acid produced by either organism. This interpretation of the data presented is at variance with the ideas of Boekhout and Ott de Vries (1). These investigators consider that the associated organisms (Aromabildner) form substances in milk from which the lactic acid bacteria produce the aroma. They base their supposition on the facts that (1) cultures of lactic acid bacteria a few days old, and accordingly coagulated, were heated at 100° C. for some time and, after cooling, inoculated with the associated type without the development of aroma and (2) old cultures of the associated organisms heated at 100° C. for ten minutes, cooled, and inoculated with lactic acid bacteria, gave an aroma after five to ten days. Their conclusion can, however, very justly be questioned for the reasons that (1) according to the data which have been presented, the associated organisms cannot produce a high volatile acidity in the presence of considerable lactic acid, either developed by *S. lacticus* or added in a sterile condition, so that in the heated lactic acid cultures used by Boek-

hout and Ott de Vries the associated type likely could not develop, and (2) the volatile acidity produced by the associated types in the milk was in part, at least, freed by the lactic acid produced when the lactic acid bacteria were later added, and this freeing of volatile acid probably resulted in the development of an aroma.

The production of a desirable starter seems to require the action of *S. lacticus* and one of the associated organisms. While *S. lacticus* is generally present in much larger numbers than the associated forms under practical conditions, it is probable that a satisfactory balance is essential. The failure to maintain such a balance is undoubtedly one of the reasons why unsatisfactory results are so frequently secured when starters are carried in practice. Altho *S. lacticus* grows well over a considerable range of temperature, 21° to 23° C. is undoubtedly best for the propagation of starters. One reason why this temperature is best is because the proper balance between the different organisms concerned is most easily maintained here. Type A, which does not grow at 37° C., cannot be expected to develop as well at temperatures approaching this as it does at 21° to 23° C.

CONCLUSIONS

1. There are at least two types of organisms associated with *S. lacticus* in starters frequently enough to be considered important. These were studied as Type A and Type B.
2. Type A can usually be isolated by direct plating and also by the method of repeated transfers on whey agar slopes.
3. Type A was repeatedly isolated from starter being carried in creameries.
4. Type A was generally present in commercial cultures.
5. The Type A and *S. lacticus* combinations were not specific, since Type A from one starter and *S. lacticus* from another gave a high volatile acidity, as did also Type A in combination with *S. lacticus* cultures from sour cream.
6. When Type A was inoculated one to four days before *S. lacticus*, a high volatile acidity was secured.
7. When Type A was destroyed by heat after a considerable period of growth and *S. lacticus* inoculated, a low volatile acidity, essentially like that produced by Type A alone, was secured.
8. When *S. lacticus* developed considerable acid before Type A was inoculated, only a low volatile acidity was obtained; this occurred when *S. lacticus* was destroyed by heat before inoculating Type A, and when it was not.
9. When Type A was added to milk in which *S. lacticus* had produced a small amount of acid and had then been destroyed by

heat, a high volatile acidity was secured. *S. lacticus* was inoculated before Type A and a high volatile acidity secured when the development of *S. lacticus* had not gone too far.

10. Type A produced a higher volatile acidity in milk to which a small amount of sterile lactic acid had been added than in milk without the acid.

11. Excessive amounts of sterile lactic acid added to milk prevented the production of a high volatile acidity by the associated organism, Type A.

12. The addition of sterile ammonium or calcium lactate to milk did not increase the volatile acidity produced by Type A.

13. Type A produced volatile acid from sterile citric acid added to milk and this suggests that the citrc acid normally present in milk is the source of the volatile acid developed when Type A is grown in pure culture in milk.

14. The volatile acidity produced by Type B alone was higher than that produced by Type A and usually was as high as that produced by a good starter.

15. When Type B was grown in combination with *S. lacticus*, a higher volatile acidity was usually produced than when Type B was grown in pure culture, but this increase can be accounted for by the volatile acid production of *S. lacticus*.

16. Type B can be isolated by direct plating.

17. Type B was found regularly in only one of the commercial cultures studied; it was occasionally found in others, however, and was also found in natural starters. Some good starters never yielded Type B.

18. The addition of sterile citric acid to milk increased the volatile acidity produced by Type B. This indicates that the citric acid normally present in milk is one of the sources of the volatile acidity produced by Type B.

19. The addition of sterile lactic acid did not significantly increase the volatile acidity produced by Type B.

20. The addition of lactates usually resulted in a decrease in the volatile acidity produced by Type B.

21. Type B produced some lactic acid in milk and it seems that a portion of this was later changed to a volatile acid.

22. The associated organisms delayed the acid development by *S. lacticus* when inoculated before *S. lacticus* and also when inoculated at the same time. This delay was evident from (1) the time required for coagulation, (2) the time elapsing between beginning and complete coagulation and (3) the acidity titrated.

23. In general both creamery and commercial cultures contained much larger numbers of *S. lacticus* than of the associated organisms. *S. lacticus* often made up 90 percent of the flora and only occasionally fell under 75 percent. In some cases the

associated types made up only from 1 to 3 percent of the flora.
24. Satisfactory starters can be prepared from mixtures of *S. lacticus* and one or both of the associated types, but not from pure cultures of *S. lacticus.*
25. The name *Streptococcus citrovorus* is proposed for Type A and the name *Streptococcus paracitrovorus* for Type B.

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The Type of Lactic Acid Produced by Starters and by the Organisms Isolated from Them

By B. W. HAMMER

From work that has recently been done it is evident that starters are not pure cultures of *S. lacticus* as has commonly been supposed.* Hammer and Bailey (2) showed that pure cultures of this organism do not produce a volatile acidity at all comparable with that produced by a satisfactory starter, and that starters generally contain an associated organism which, when grown in combination with *S. lacticus*, yields a volatile acidity essentially the same as that produced by a good starter. Storch (5) found that organisms which he calls *x* bacteria are present in starters and that these play a part in the production of a good product. Boekhout and Ott de Vries (1) reported the isolation from starters of organisms that, in combination with lactic acid bacteria, produce the characteristic aroma.

The proof that starters are not pure cultures of *S. lacticus* can be arrived at in an entirely different way by determining the type of lactic acid produced in a starter and comparing it with the type produced in milk by pure cultures of *S. lacticus*. Data on this method of proof and also on the type of lactic acid produced when the associated organisms** are combined with *S. lacticus* are presented in this bulletin.

METHODS

The zinc lactates for the determination of the isomeric modification of lactic acid produced were prepared as follows:

The fermented milk was heated in a water bath to facilitate the separation of the whey and then thrown on a filter paper. In a few instances where there was comparatively little acid in the milk, N/1 H_2SO_4 (25 cc. to 750 cc. of milk) was added before the heating to facilitate the separation of the whey and to liberate the lactic acid. The whey, to which N/1 H_2SO_4 was added at the rate of about 5 cc. per 100 cc. of whey, if none had been added to the milk, was evaporated to a comparatively small volume and plaster of Paris added, usually at the rate of 15 grams to 100 cc. of the original whey, and well stirred in to take up the remaining water. By breaking up the mass soon after setting

* For one of the more recent and very definite statements see Guthrie, E. S. The Book of Butter. 1918. p. 111.

** The associated organisms found in starters have been divided into Types A and B. Type A has been named **Strep. citrovorus** and Type B, **Strep. paracitrovorus.** (Res. Bull. 63, Ia. Agr. Exp. Sta.)

had begun, a hard lump was prevented from forming and the material kept in a condition suitable for the subsequent handling. The lactic acid was extracted by putting the whey and plaster Paris combination into a thimble and extracting with ether for approximately 25 hours in an apparatus in which the solvent dripped on the material in the thimble. After transferring the ether and the dissolved material to a beaker, the ether was allowed to evaporate, water added, and then $ZnCO_3$ in excess.

The material was boiled with animal charcoal to decolorize, filtered, and the insoluble portion washed well with hot water. The filtrate was evaporated down and allowed to crystallize; after the first crop of crystals had been removed, the filtrate was again evaporated and another crop obtained. Every effort was made to crystallize the zinc lactate as completely as possible, because the difference in solubility of the zinc salts of the active and inactive acids would result in an unrepresentative preparation if only a part of these were secured from a mixture. At best there is likely to be some error due to disproportionate amounts being lost when mixtures are being crystallized, but this cannot be very serious if care is taken to carry the crystallizations to as near completion as possible. The zinc lactates secured were recrystallized at least once and sometimes twice, dried and finely ground, after which they were allowed to dry in air to constant weight.

In determining the type of lactic acid, the percent water of crystallization was largely relied on. This was secured by drying the prepared salts to practically constant weight at 110°C. Some of the preparations were then run in a polariscope but, since in most instances the presence of inactive lactic acid was the principal point to be determined, the rotation was tested with only a part of the preparations.

In determining the percent acid in milk, a 5 or 10 gram sample was titrated with N/10 NaOH, using phenolphthalein as an indicator, and the results calculated as the percent lactic acid.

In a few instances volatile acid determinations were made according to the method followed by Hammer and Bailey (2).

RESULTS OBTAINED

TYPE OF LACTIC ACID PRODUCED BY *S. LACTICUS*

The work of a number of investigators has shown that *S. lacticus* produces *d* lactic acid. Heinemann (3), who also gives a review of the literature, studied 11 cultures and found *d* acid at both 37°C. and room temperature. Suzuki, Hastings and Hart (6) found that *B. lactis acidi* (*S. lacticus*) produced ac-

tive acid in a lactose solution. Orla-Jensen (4), in a recent report on the lactic acid bacteria, states that his genus Streptococcus, to which *S. lacticus* (Orla-Jensen's *S. lactis*) belongs, forms, from all carbohydrates, pure *d* lactic acid. In the work herein reported, as well as in unreported trials that were previously carried out, *S. lacticus* was consistently found to produce *d* acid. It seems accordingly to be well established that *S. lacticus* produces *d* lactic acid only, at least under the usual conditions of growth.

TYPE OF LACTIC ACID PRODUCED IN STARTERS

In order to determine the type of lactic acid produced in starters carried under practical conditions, a number of starters that had been carried in a creamery were examined by the method previously outlined. The results obtained are presented in table I.

From the values given in table I for the percent of water of crystallization in the zinc lactates, it is evident that the starters did not yield pure *d* lactic acid, but a mixture of active and inactive acid, since the values were over the 12.89 percent theoretical for active acid, but did not reach the 18.18 percent theoretical for inactive acid; they exceeded 14 percent in all cases but one and in this the value was almost 14 percent. Because of the abundance of *S. lacticus* in starters, it would be expected that the active acid would be of the *d* type, and this was confirmed in those cases in which the rotations were run, since the zinc salt gave an *l* value. The results presented, which are extensively confirmed in later tables, indicate that starters as carried under factory conditions do not represent practically pure cultures of *S. lacticus*, as has been frequently stated, because if they did, pure *d* lactic acid should be secured from them.

TABLE I—TYPE OF LACTIC ACID PRODUCED IN STARTERS CARRIED UNDER PRACTICAL CONDITIONS

Original Inoculating Material from	Number of Transf's	Acidity in Percent	Results on $(CH_2CHOHCOO)_2Zn$			
			Percent H_2O			Specific Rotation
			A	B	Average	
Manufacturer A		.92	13.95	14.01	13.98	
Manufacturer A			15.14	15.07	15.105	
Manufacturer A		.92	14.50	14.44	14.47	
Manufacturer A		.96	14.33	14.17	14.25	
Manufacturer A		.87	14.33	14.51	14.42	
Manufacturer G	7	.93	14.11	14.08	14.095	*l* 5.93
Manufacturer H	8	.95	13.98	14.07	14.025	*l* 6.77
Manufacturer F	9	.96	14.10	14.20	14.15	*l* 5.82
Natura Starter	11	1.63	15.20	15.08	15.14	*l* 5.85

The results presented in table I were secured on starters that had developed a considerable amount of acid, altho in all cases they had been ripened only over night, this being the usual practice in the factory from which they were obtained. In order to see what type of lactic acid was present in starters at low acidities, the results given in table II were secured. The material that was studied was obtained by inoculating two flasks containing the same amount of milk from the same lot with equal amounts of starter and holding at room temperature; the flasks were then studied at very different acidities.

The data given in table II show that at the low acidities the starters sometimes contained practically only active acid and sometimes certain amounts of inactive along with the active acid. At the high acidities there was always some inactive acid, the water of crystallization of the zinc lactates being over 14 percent in all cases; these results are in agreement with those reported in table I. In those starters in which there was little or no inactive acid at the low acidity, this type of acid was always evident at the high acidity, but where there was inactive acid at the low acidity the percent did not change greatly as the amount of total acid increased; in two cases there was actually a decrease in the percent of inactive acid. In one instance an acid was secured which the water of crystallization of the zinc salt showed to be almost entirely an inactive acid. The culture used for inoculating in this case came from a manufacturer whose product usually gave an acid of this character, as will be shown in some of the later tables; the culture differed from most cultures in that it contained an associated organism of the B type.

TYPE OF LACTIC ACID PRODUCED BY MIXTURES OF *S. LACTICUS* AND ASSOCIATED ORGANISMS

Since starters do not yield a pure *d* lactic acid, as would be the case if they were essentially pure cultures of *S. lacticus*, it would be expected that the type of lactic acid produced would be influenced by the associated organisms, inasmuch as these have been shown to be of importance in starters. Data showing the type of lactic acid produced by mixtures of *S. lacticus* and associated organisms inoculated at the same time, and by *S. lacticus* alone, are presented in table III. The data should be divided at the double line, since the figures above this line were obtained with the A type of associated organism and those below with the B type. The cultures of *S. lacticus* alone yielded what should be considered pure active acid, when judged by the water of crystallization of the zinc salts. The values are generally slightly higher than the theoretical, due in all probability

TABLE II—TYPE OF LACTIC ACID PRODUCED IN STARTERS AT DIFFERENT ACIDITIES

Room Temperature Incubation

Condition of milk Inoculated	Low Acidities						High Acidities				
	Age in Days	Acidity in percent	Results on $(CH_3CHOHCOO)_2Zn$ Percent H_2O A	B	Average	Specific Rotation	Age in Days	Acidity in percent	Results on $(CH_3CHOHCOO)_2Zn$ Percent H_2O A	B	Average
Pasteurized	Under 1	.64	14.03	14.00	14.015		3	.97	14.31	14.58	14.445
Sterile	Under 1	.68	15.05	15.17	15.11		3	1.11	14.80	14.79	14.795
Sterile	Under 1	.52	17.21	17.26	17.235		2	.86	17.11	17.14	17.125
Sterile	Under 1	.69	13.39	13.54	13.465		2	1.03	14.36	14.39	14.375
Sterile	Under 1	.63	12.99		12.99	l 8.34	3	1.03	14.52	14.67	14.595
Sterile	Under 1	.59	13.18	13.18	13.18		4	1.05	13.98	14.10	14.04

TABLE III—TYPE OF LACTIC ACID PRODUCED IN MILK BY *S. LACTICUS* ALONE AND BY *S. LACTICUS* AND ASSOCIATED ORGANISMS INOCULATED TOGETHER

S. lacticus alone					**S. lacticus** and associated organisms inoculated together							
No. **S. lacticus** Inoculated	Days Held	Results on $(CH_3CHOHCOO)_2Zn$ Percent H_2O A	B	Average	Assoc. Org. No.	Inoculated Type	Days Held	Results on $(CH_3CHOHCOO)_2Zn$ Percent H_2O A	B	Average	Specific Rotation
1	8	12.98	13.07	13.025	1	A	8	15.62	15.83	15.725	
2	8	12.93	12.90	12.915	2	A	8	18.16	17.96	18.06	
3	8	12.88	12.91	12.895	3	A	8	16.60	16.36	16.48	
4	7	13.23	13.20	13.215	4	A	7	17.99	18.07	18.03	
6	7	13.11	13.11	13.11	6	A	7	13.77	13.66	13.715	
8	5	13.29	13.25	13.27	2	A	5	13.85	14.00	13.925	
10	5	12.97	13.05	13.01	1	A	5	13.78	13.73	13.755	
10	4	13.23	13.26	13.245	7	A	4	14.16	14.27	14.215	l 4.47
					14	A	7	16.07	16.03	16.05	l 5.08
6*	7	13.11	13.11	13.11	107	B	7	16.41	16.31	16.36	
7**	7	12.94	13.07	13.005	108	B	7	18.15	18.03	18.09	
9	7	13.04	13.01	13.025	108	B	7	16.96	17.05	17.005	

* Values for **S. lacticus** 6 alone repeated from above.

** Slow curdling culture of **S. lacticus.**

to hygroscopic moisture. Altho rotations were not run on the zinc salts, it can safely be assumed from the work of others and from unreported data obtained in this laboratory, that these pure active acids were of the *d* type. The values secured for the water of crystallization of the zinc salts obtained with the mixtures of organisms, show that in all instances there was some inactive acid present, while in a few cases almost a pure inactive acid was produced. The two salts on which rotations were run gave an *l* value, showing that the active acid was of the *d* type. The percent of inactive acid secured with Type A in association with *S. lacticus* was extremely variable while with Type B it was quite high in each of the three trials. The data presented in table III show that the presence of the associated organisms influences decidedly the type of lactic acid produced.

The variations in the percent of inactive acid secured in the different trials reported in table III, especially with the associated organisms of the A type, would probably be ascribed to the variations in the numbers of the associated organisms and their opportunity for growth. These organisms cannot grow if the milk is too acid and in all probability in mixtures with *S. lacticus* the activity of the associated organisms is stopped when the acidity reaches a certain point. In order to determine the effect of large numbers of associated organisms, comparisons were made of the type of lactic acid produced in mixtures of these forms and *S. lacticus* inoculated at the same time, with the type developed in mixtures made by inoculating the associated organism some time before *S. lacticus*, so that the former would be present in unusually large numbers. The results obtained are presented in table IV. The data should again be divided at the double line, those above representing the A type of associated organism and those below the B type. The results secured when the two organisms were inoculated at the same time are essentially the same as those reported in table III. When the associated organism was present in large numbers, as a result of being inoculated from one to three days before *S. lacticus*, the lactic acid present in all but one of the seven trials was essentially pure inactive acid, as is shown by the water of crystallization of the zinc lactates. The values for five of these six salts are slightly higher than the theoretical for the zinc lactate of inactive acid, and this again is presumably due to hygroscopic moisture. The data given in table IV show that the numbers of the associated organisms influence materially the amount of inactive acid formed; that is, with an increased activity of the associated organisms there is a greater amount of inactive acid produced.

TABLE IV—TYPE OF LACTIC ACID PRODUCED IN MILK BY *S. LACTICUS* AND THE ASSOCIATED ORGANISMS INOCULATED AT THE SAME TIME COMPARED WITH THE ACID PRODUCED BY THE TWO WHEN THE ASSOCIATED ORGANISMS WERE INOCULATED BEFORE *S. LACTICUS*

Room Temperature Incubation

Organisms Used			Days Held	Organisms inoculated at the same time				Associated organisms inoculated before S. lacticus				
				Results on $(CH_3CHOHCOO)_2Zn$						Results on $(CH_3CHOHCOO)_2Zn$		
Assoc. Org.		S. lacticus No.		Percent H_2O			Specific Rotation	Days Assoc. Org. was inoculated before S. lacticus	Days held after inoculating S. lacticus	Percent H_2O			Specific Rotation
No.	Type			A	B	Average				A	B	Average	
13	A	9	4	13.93	13.83	13.88	*l* 6.85	2	4	18.25	18.31	18.28	0
16	A	9	4	13.36	13.30	13.33	*l* 5.67	2	4	18.34	18.39	18.365	0
13	A	9	5	14.06	14.12	14.09	*l* 6.40	1	5	18.28	18.29	18.285	0
14	A	9						3	4	17.23	17.25	17.24	
106	B	9	5	15.13	15.30	15.215	*l* 5.29	1	5	18.28	18.36	18.32	0
111	B	9						1	5	18.22	18.31	18.265	
113	B	9						1	5	18.18	18.16	18.17	0

The associated organisms of the A type have been found capable of producing volatile acid from lactic acid, either added to milk in a sterile condition or produced in milk by *S. lacticus* whose activity was stopped by heating so that excessive amounts of acid would not be present. In order to see what type of lactic acid resulted when S. *lacticus* was allowed to develop a small amount of acid in milk, and then killed with heat before the associated organisms were added, the data presented in table V were secured.

The figures represent three different series and include volatile acid determinations made at the end of the incubation period. In series 1 the addition of an associated organism changed the active acid produced by *S. lacticus* to an almost pure inactive type and at the same time produced considerable volatile acid. The production of inactive acid in series 2 was only slight and this was probably due to the presence of acid, after the growth of *S. lacticus*, in sufficient amount to inhibit the development of the associated organism. This idea is substantiated by the comparatively low volatile acid production in this series. In series 3 the active acid produced by *S. lacticus* was practically all changed over to the inactive type by the four different organisms tried, and with the three of these for which figures are given, volatile acid was produced in considerable amounts.

In table V the values representing the water of crystallization of both the pure *d* and the pure inactive acids are generally higher than the theoretical and this is again in all probability due

TABLE V—TYPE OF LACTIC ACID PRODUCED WHEN *S. LACTICUS* WAS GROWN IN MILK AND KILLED AND THEN THE ASSOCIATED ORGANISMS ADDED

Room Temperature Incubation

Series No.	S. lacticus culture No.	Days S. lacticus was grown before heating to kill org.	Assoc. Org. used in reinoculating		Days held after reinoculation	Results on $(CH_3CHOHCOO)_2Zn$				Volatile Acidity
			No.	Type		Percent H_2O			Specific Rotation	
						A	B	Aver.		
1	9	1	1	A	6	17.43	17.36	17.395		34.6*
		1	None			13.19	13.32	13.255		14.3
2	8	1	7	A	7	13.84	13.89	13.865		25.2
		1	None			13.07	13.18	13.125		7.8
3	9	1	15	A	6	18.55	18.64	18.595	0	41.4
		1	16	A	6	18.37		18.37	0	41.3
		1	121	B	6	18.33	18.33	18.33	0	41.7
		1	126	B	6	18.25	18.22	18.235	0	
		1	None			13.19	13.12	13.155	8.82	8.2

* These values represent the cc. N/10 NaOH required to neutralize the first liter of distillate obtained when a 250 gram sample of milk was distilled with steam after the addition of 15 cc. of approximately N/1 H_2SO_4. See Hammer and Bailey (2).

to hygroscopic moisture. The results presented indicate that when the associated organisms were grown in milk containing active acid of the *d* type, the acid resulting was partly or entirely of the inactive type.

INFLUENCE OF TEMPERATURE ON THE TYPE OF LACTIC ACID PRODUCED BY STARTERS

One of the most important differences between associated organisms of the A and of the B types is the inability of the former to grow at 37°C. The presence or absence of Type B would accordingly be expected to influence materially the type of lactic acid produced by a starter carried at 37°C. In order to try out this idea, a number of starters were inoculated into two flasks of sterile milk, one being held at 37°C. and the other at room temperature. After incubation, the type of lactic acid present was determined in the usual way. The results secured are given in table VI.

Starters containing associated organisms of the A type regularly produced significant amounts of inactive acid at room temperature, but at 37°C. they produced very little if any. In the few instances when there may have been a small amount of inactive acid at 37°C. (altho the value slightly greater than the theoretical for active acid is probably due to hygroscopic moisture), the amount was very definitely less than at room temperature. Starters containing associated organisms of the B type produced inactive acid at both temperatures, but at 37°C. the lactic acid was apparently all inactive (the one value higher than the theoretical is probably due to hygroscopic moisture), while at room temperature it was not, altho in two of the three trials there were considerable amounts of inactive acid.

Starters containing the B type of associated organism differed decidedly from those containing only the A type in that they produced inactive acid in large amounts at 37°C. The data reported in table VI show another important difference; when Type B was present in a starter a higher acidity was produced at 37°C. than at room temperature, but when Type A was present without Type B the higher acidity was produced at room temperature.

MECHANISM OF THE FORMATION OF THE INACTIVE ACID

In considering the production of inactive acid in starters, or in mixtures in milk of *S. lacticus* and the associated organisms, the most plausible assumption is that since *S. lacticus* produces *d* acid, the associated organisms must produce *l* acid. The two types of associated organisms will be considered separately in dealing with this explanation.

TABLE VI—TYPE OF LACTIC ACID PRODUCED BY STARTERS IN STERILE MILK AT DIFFERENT TEMPERATURES

Inoculating Material	Type of Assoc. Org. present	Room Temperature						37 degrees centigrade					
		Age in Days	Acidity in percent	Results on $(CH_3CHOH\ COO)_2Zn$ Percent H_2O A	B	Av.	Specific Rotation	Age in Days	Acidity in percent	Results on $(CH_3CHOH\ COO)_2Zn$ Percent H_2O A	B	Av.	Specific Rotation
Creamery Starter	A	4		14.69	14.55	14.62		4		13.38	13.18	13.28	
Commercial Culture	A	5		15.14	15.30	15.22		5		13.22	13.20	13.21	
Creamery Starter	A	3	1.11	14.80	14.79	14.795		3	.67	13.31	13.31	13.31	
Creamery Starter	B	2	.86	17.11	17.14	17.125		2	1.03	18.19	18.23	18.21	
Creamery Starter	A	2	1.03	14.36	14.39	14.375		2	.72	13.66	13.46	13.56	
Commercial Culture	B	2		13.81	13.86	13.835	[6.75	3		18.78	18.76	18.77	0
Commercial Culture after 6 generations in sterile milk	A	4	1.20	13.36		13.36		4	.81	13.03		13.03	
Commercial Culture after 6 generations in sterile milk	A	5		14.33	14.31	14.32	[5.65	5		13.76	13.76	13.76	
Commercial Culture after 6 generations in sterile milk	A	5	1.20	14.28	14.20	14.24	[6.93	5	.74	13.08	13.25	13.165	[7.50
Commercial Culture after 6 generations in sterile milk	B	3	1.04	16.52		16.52		3	1.08	18.18		18.18	
Commercial Culture after 6 generations in sterile milk	?	3	.90	14.55	14.37	14.46	[5.60	3	.67	13.88		13.88	

Type B usually produced considerable amounts of acid in milk, the acidities observed varying from .39 to .77 percent, calculated as lactic acid, with the most of the cultures not coagulating milk. Type B in general produced less total acidity than *S. lacticus* and considerably more volatile acidity. Accordingly, if lactic acid were the non-volatile acid, it would be expected to be present in smaller amounts than with *S. lacticus.* In order to determine whether or not Type B produced lactic acid and if so, its rotation, the results presented in table VII were secured. These data show that the values for the water of crystallization of the zinc salts of the acid produced are such that they correspond to the theoretical for zinc lactate where there is some active and some inactive acid. The sample showing 17.76 percent water of crystallization yielded 33.76 percent ZnO on a water-free basis, which suggests lactic acid, since the theoretical ZnO value for anhydrous zinc lactate is 33.46 percent. For the preparations on which rotations were run, the results indicate lactic acid, and since the zinc salts wer *d*, the free acids were of the *l* type.

The type of lactic acid produced by associated organisms of the B type and *Bact. aerogenes* when the associated organism was inoculated before the other organism, is shown in table VIII. The culture of *Bact. aerogenes* used was isolated from an outbreak of ropy milk and produced lactic acid that was largely inactive,* as is shown by the values for the percent water of crystallization and ZnO given in table VIII. When the associated organisms were inoculated first and *Bact. aerogenes* later, zinc lactates were obtained whose percent water of crystallization indicated essentially an active acid, and since the rotations were *d*, the lactic acid was of the *l* variety. The surprising thing is that there was no evidence of appreciable amounts of inactive acid after the growth of *Bact. aerogenes,* which, judged by the development of gas, was very vigorous.

The data presented show that Type B produced lactic acid which varied from largely active to largely inactive, with the excess of active acid of the *l* type. The data also suggest (but

TABLE VII—TYPE OF LACTIC ACID PRODUCED IN MILK BY ASSOCIATED ORGANISMS OF THE B TYPE

Room Temperature Incubation

Organism No.	Age in Days	Results on $(CH_3CHOHCOO)_2Zn$		
		Percent H_2O	Specific Rotation	
113	8	17.76		
117	8	14.58	d	6.44
118	10	13.11	d	7.65

* **Bact. aerogenes** is commonly considered to produce *l* acid.

TABLE VIII—TYPE OF LACTIC ACID PRODUCED IN MILK BY *BACT. AEROGENES* ALONE AND BY ASSOCIATED ORGANISMS OF THE B TYPE COMBINED WITH *BACT. AEROGENES*

Associated Organism		Bact. aerogenes Inoc.	Date examined	Temp. of incubation Degrees C.	Res. obt'd on $(CH_3CHOH\ COO)_2Zn$				Specific Rotation	Percent ZnO
					Percent H_2O					
No.	Date of inoc.				A	B	Av.			
		3-30	4-2	37	18.12		18.12			33.00
		4-6	4-9	37	17.86		17.86			33.26
113	3-25	3-30	4-6	20	12.92	12.89	12.905	d	7.25	
113	3-30	4-8	4-13	37	13.19	13.16	13.175	d	5.88	
115	4-20	4-26	5-3	37	13.11	13.10	13.105	d	6.00	

cannot be considered to prove) that the *d* acid can be changed to *l* acid by the Type B organism, since first, it seems impossible that the excess of *l* acid produced by Type B could alone yield inactive acid with the *d* acid produced by *S. lacticus* and second, the inoculation of Type B several days before *Bact. aerogenes* resulted in practically only active acid of the *l* type, altho *Bact. aerogenes* alone gave largely inactive acid.

Type A produced very little total acidity in milk under ordinary conditions and of this, a much larger percentage was volatile than was the case with the total acidity produced by *S. lacticus*. A number of milk cultures of Type A in 750 cc. amounts were examined by the method used for the preparation of zinc lactate, but in all cases the amount of salt obtained was too small for even a reasonably careful study. In one instance three such preparations were combined in an impure condition and, without recrystallizing, the percent water of crystallization and ZnO determined. The results obtained suggested lactic acid of the inactive type, but the values secured differed considerably from the theoretical.

The data presented in table V contain evidence of interest in connection with the formation of inactive acid. The flasks of milk containing *S. lacticus* were heated to kill the inoculated organism at a time when coagulation had definitely begun but was still incomplete, the acidity probably being about .55 to .65 percent calculated as lactic acid. In series 1 and 3 Type A, when inoculated, yielded practically all inactive acid, together with a high volatile acidity. The total acidity produced in the milk by *S. lacticus* of which the lactic acid was of the *d* type was much greater in amount than the total acidity which Type A was found capable of producing and, moreover, a much smaller percentage of the former is volatile than is the case with the acid produced by Type A. This analysis of the data suggests that even if the non-volatile acid produced by Type A were lactic acid of the *l* type, which is probably not the case,

the amount would be much too small to give inactive acid almost entirely when combined with an acidity of from .55 to .65 percent produced by *S. lacticus*.

The results obtained suggest, but do not prove, that Type A has the ability to change *d* acid to the *l* form, because it seems that the amount and character of the non-volatile acid produced by Type A are such that it would not yield inactive lactic acid when combined with considerable amounts of *d* acid produced by *S. lacticus*.

The proof of whether or not the associated Types A and B can change *d* to *l* acid cannot readily be secured until a medium free from citric acid* and carbohydrates, in which the organisms grow well, is obtained. Under such conditions lactic acid can be added and later recovered without including acid developed by the associated types from constituents of the medium.

DISCUSSION OF RESULTS

The data obtained on the type of lactic acid produced show that starters are not practically pure cultures of *S. lacticus* and that the associated organisms of both Types A and B are undoubtedly important in producing the lactic acid other than the *d* type formed by *S. lacticus*.

The results secured suggest, but do not prove, that the associated organisms of both types have the ability to change *d* to *l* lactic acid. If this is found actually to be the case, it will represent an unusual type of bacterial activity.

The type of lactic acid produced by starters and by mixtures of *S. lacticus* and associated organisms in milk, is of interest in connection with the results obtained by Suzuki, Hastings, and Hart (6) in their study of cheddar cheese. These investigators found the usual type of lactic acid in cheddar cheese to be inactive, and they secured a mixture of active and inactive acids when a bit of cheese was used to inoculate a lactose solution, but active acid when *B. lactis acidi* (*S. lacticus*) or a starter was used for inoculating. It is difficult to explain the difference in the results obtained by them when a bit of cheese was used for the inoculation of the lactose solution and when starter was used, but it seems probable that the associated organisms play a part in the formation of the inactive lactic acid in cheese.

CONCLUSIONS

1. Starters carried under practical conditions contained some inactive lactic acid; accordingly they cannot be considered to

* The associated types produce volatile acid from citric acid. Ia. Exp. Sta. Res. Bull. 63.

represent practically pure cultures of *S. lacticus*, since this organism produces only *d* acid.

2. At low acidities starters sometimes contained practically only active lactic acid and sometimes some inactive with the active. At high acidities there was always some inactive acid.

3. With mixtures of *S. lacticus* and the associated organisms, some inactive lactic acid was always present. With associated organisms of the A type the percentage of inactive acid was extremely variable, while with Type B it was generally quite high.

4. With an increased activity of the associated organisms, as occurred when they were inoculated some time before *S. lacticus*, there was a greater amount of inactive acid produced.

5. When the associated organisms were grown in milk containing some *d* lactic acid as a result of the activity of *S. lacticus*, the *S. lacticus* organisms having been killed with heat. the acid resulting was partly or entirely of the inactive type.

6. Starters containing associated organisms of the B type differed from those containing only the A type in producing inactive acid in large amounts at 37°C. and in yielding a higher percent acidity at 37°C. than at room temperature.

7. Type B produced lactic acid, some of which was usually inactive.

8. An analysis of the data obtained suggests that the associated organisms of both Types A and B may be able to change *d* lactic acid to the *l* form, but does not supply proof of this.

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